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STIMULATION OF RAT ADRENOCORTICAL SECRETION BY A FACTOR PRESENT IN HUMAN URINE

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ABSTRACT

Production of corticosteroids by the rat adrenal gland has been studied in vitro. Both a commercial and a highly purified preparation of ACTH stimulated the secretion of "corticosterone" and aldosterone *in vitro*. The "corticosterone" fraction was increased more than aldosterone. Addition of the non-ultrafilterable fraction of human urine to the incubate stimulated the secretion of aldosterone more than "corticosterone." Several possible interpretations of this effect are discussed.

THE rat adrenal gland has been demonstrated to produce corticosterone and aldosterone *in vitro* (1, 2). Stimulation of this production by ACTH has also been shown (1, 2). The technique, therefore, offers an opportunity to study factors directly influencing either glucocorticoid or mineralocorticoid secretion. In the present study, the non-ultrafilterable fraction of human urine has been assayed for its effect on adrenocortical secretion.

METHODS

Adrenal glands from 60 to 80 rats were trimmed of fat, bisected, weighed, and apportioned equally to the control and experimental flasks. Each flask received one-half of an adrenal from each rat. For 25 mg. of tissue, 1 cc. of Krebs-Ringer bicarbonate buffer with glucose, 200 mg. per 100 cc., was added to the flasks. The flasks were incubated in a Dubnoff metabolic incubator at 37.5° C in an atmosphere of 95% O_2 and 5% CO_2. After one-half hour of incubation, the medium was poured off and discarded. An equal quantity of fresh medium, containing the material to be tested, was added and incubation was continued for two hours. Each medium was extracted with several volumes of redistilled chloroform. The chloroform extract was chilled, washed once with cold 0.05N NaOH, and twice with cold distilled water, dried with anhydrous sodium sulfate and evaporated *in vacuo* at 40° C. The residue was applied to paper and chromatographed

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in the chloroform/formamide system, using reference standards of cortisol, cortisone, and corticosterone. A contact photoprint of the chromatogram was made using a G.E. Sterilamp as a light source. The fractions indicated by the major ultraviolet-absorbing bands and by the reference standards were eluted with ethanol. Formamide was removed by partitioning the residue between water and chloroform. In some instances, fractions were rechromatographed in other systems. The ultraviolet absorption peak near 240 mμ was used to detect and measure alpha, beta-unsaturated ketones. Soda fluorescence (3), reduction of blue tetrazolium (3), and Zimmerman reaction (4) were measured in several fractions. Aldosterone was estimated by bioassay in adrenalectomized rats (5), using a ratio of activity of aldosterone to desoxycorticosterone acetate of 36.5 to 1.

Human urine, preserved by refrigeration, was ultrafiltered through a 2% collodion membrane (6). The protein was eluted from the membrane by the method of Rigas and Heller (7), using isotonic saline. Urine "protein" A and B were lyophilized pools of the urine protein obtained from 100 and 75 patients, respectively. Urine "protein" C and E were each a 48-hour pool from two individuals. Urine "protein" F was a pool from 20 normal adults.

Urine "protein" A_D was a quantity of urine "protein" A which was dialyzed in the cold against distilled water.

RESULTS

Some characteristics of the fractions consistently separated by paper partition chromatography are presented in Table 1. Although not conclusively identified, corticosterone and aldosterone are presumed to be present.

TABLE 1. CHROMATOGRAPHIC BEHAVIOR AND SPOT TESTS OF ADRENAL SECRETION

System: Chloroform-foramide		Rechromatographed in:
Fraction I *Rf:*		*Bush C*
One narrow band: U-V absorption (234 mμ)	——→	One band, distinctly more polar than cortisol.
Slightly more polar than cortisol		Has soda fluorescence. Reduces blue tetrazolium slowly. Negative Zimmermann reaction
Fraction II *Rf:*		
Narrow, faint band of U-V absorption		
Runs near cortisone.		
Reduces blue tetrazolium		
Strong mineralcorticoid on bioassay presumed to be aldosterone		
Fraction III–IV *Rf:*		*Toluene-propylene glycol*
		Two bands of U-V absorption (240 mμ)
	——→	*Fraction III* = More polar than corticosterone
Broad band near corticosterone "B fraction"		Does not reduce blue tetrazolium;
		Has soda fluorescence. Negative Zimmermann reaction
		Fraction IV = Presumed to be corticosterone.
		Reduces blue tetrazolium; has soda fluorescence
Fraction V *Rf:*		
Broad band near solvent front		

TABLE 2. ACTH

Experiment	Dose/Flask	μg. aldosterone eqv./100 mg. tissue/2 hrs.	%Δ	μg. B eqv. /100 mg. tissue/2 hrs.	%Δ
Control		0.15	+135	8.3	+422
ACTH (Armour)	4.0 units	0.36		43.3	
Control		0.20	+ 65	3.9*	+254
ACTH (Armour)	3.8 units	0.33		13.8*	
Control		0.25	− 7	6.7	+ 75
ACTH† (C. H. Li)	40 μg.	0.23		11.7	
Control		0.13	+112	6.6	+435
ACTH† (C. H. Li)	200 μg.	0.28		35.4	

Mean diff. test-control 0.12∓0.04 (S.E. of mean). Significant at $P < .05$ (t test).
* Only Corticosterone measured.
† Sheep ACTH.

EFFECT OF ADRENOCORTICOTROPHIN (ACTH)

Table 2 contains data from two experiments with lyophilized bovine ACTH (Armour) and two with a highly purified sheep ACTH prepared by C. H. Li and co-workers (8). The "B fraction" represents the sum of the two components into which the band moving with corticosterone in the chloroform/formamide system separates when rechromatographed in toluene/propylene glycol. Both of these components appeared in increased amount when ACTH was added. Although secretion of aldosterone and the "B fraction" is usually increased by ACTH, the increase in "B fraction" is greater both absolutely and relatively.

EFFECT OF NON-ULTRAFILTRABLE FACTOR OF HUMAN URINE

Results of experiments in which urine "protein" was added to the media are presented in Table 3. Secretion of aldosterone is regularly increased by addition of the urine "protein." Although the degree of effect varies markedly among experiments, the effect is consistently in the same direction and highly significant ($P < .005$). Moreover, the pattern of corticosteroid secretion is different from that noted with ACTH. Aldosterone secretion is consistently stimulated to a greater degree than the "B fraction."

The variation in the values of the control adrenals among experiments is large. No attempt was made to control weight, sex of rats, or seasonal factors from experiment to experiment. This may well account for the variation. However, in an individual experiment, the adrenals of the control and experimental flasks were relatively homogeneous in that each flask contained one half of an adrenal from every rat and the same number of left and right adrenal halves.

Four different pools of urine "protein" showed some activity. In one experiment a dose-response effect could not be established. Possibly, the lowest dose of urine protein used produced a maximal stimulation. There

TABLE 3. HUMAN URINE PROTEIN

Experiment	Dose/Flask	μg. aldosterone eqv./100 mg. tissue/2 hrs.	%Δ	μg. B. eqv./100 μg. tissue/2 hrs.	%Δ
Control		0.08			
Urine Protein A	20 hrs.	0.22	+166	6.0	
Control		0.33		3.6	
Urine Protein A	20 hrs.	0.43	+ 30	4.2	+17
Control		0.085		5.7	
Urine Protein A	5 hrs.	0.26	+205	7.0	+22
Urine Protein C	12 hrs.	0.31	+261	7.7	+35
Control		0.18		7.2	
Urine Protein A	17 hrs.	0.47	+263	7.8	+08
Urine Protein A_D	17 hrs.	0.65	+365	8.0	+11
Control		0.72		7.4	
Urine Protein A	5 hrs.	1.20	+ 66	6.6	−11
Urine Protein A	10 hrs.	0.86	+ 19	7.6	+ 3
Urine Protein A	20 hrs.	0.86	+ 19	7.8	+ 5
Control		0.16		3.9	
Urine Protein B	5 hrs.	0.23	+ 43	4.7	+20
Urine Protein E	10 hrs.	0.26	+ 64	4.9	+25

Test-control mean diff. $= 0.19 \pm 0.045$ (S.E. of mean). $P < .005$ by t test.

was no obvious explanation for the very high control level of aldosterone secretion in this experiment. The urine "protein" which had been dialyzed against distilled water showed activity greater than before dialysis.

CONTROL EXPERIMENTS

To study the specificity of this effect, several different experiments were carried out. These are recorded in Table 4. In the first experiment, a 20-hour dose of urine "protein" A was added to a flask containing incubation media without adrenals. The flask was incubated and processed in the usual manner. No aldosterone was detected by bioassay, nor other cortico-

steroids by ultraviolet absorption, blue tetrazolium reaction and soda fluorescence. This eliminated the possibility that aldosterone bound to the urine protein was being added to the media.

In the second experiment, a quantity of urine "protein" subjected to prolonged boiling was added to a flask with rat adrenals. An equal quantity of untreated urine "protein" was added to another flask. Three pools of urine "protein" were studied in this fashion. In each instance, the untreated urine "protein" promoted a greater output of aldosterone than the boiled urine "protein." In the two experiments with urine "protein" A and urine "protein" F, this increment was outside the 95% confidence limits of the bioassay.

Another experiment shows the results of addition of purified beef growth hormone and highly purified pig melanocyte-stimulating hormone to the incubation media of rat adrenal glands. In the dose used, it was not shown

TABLE 4. CONTROL EXPERIMENTS

Experiment	Dose/Flask	μg. aldosterone /100 mg. tissue /2 hrs.	%Δ	μg. B. fraction /100 mg. tissue /2 hrs.	%Δ
Urine Protein A	20 hrs. S adrenals	0		0	
Boiled Urine Protein A	20 hrs.	0.204		4.5	
Urine Protein A	20 hrs.	0.296*	+45	3.7	−18
Control		0.157		3.9	
Boiled Urine Protein B	5 hrs.	0.190	+21	5.3	+36
Urine Protein B	5 hrs.	0.225	+43	4.7	+20
Control		0.131		—	
Boiled Urine Protein F	17 hrs.	0.150	+14	—	
Urine Protein F	17 hrs.	0.386	+197	—	
Control		0.250		6.7	
Growth Hormone	400 μg.	0.207	−17	8.3	+24
MSH	700 μg.	0.313	+25	9.2	+49
Control		0.094		8.1	
Albumin	40 mg.	0	—	6.6	−18
Gelatin	40 mg.	0	—	4.4	−56

* Assay result was above upper limit of dose response curve. Therefore, at least this quantity of aldosterone was present.

that either hormone affected the secretion of aldosterone significantly.

Gelatin, U.S.P., and four-year old human serum albumin, prepared by Cutter Laboratories, did not stimulate aldosterone production and appeared to depress "corticosterone" secretion.

DISCUSSION

In the present studies, the yield of aldosterone was much less than that reported by Giroud *et al.* (2). The yield of corticosterone, which comprises approximately half of the B fraction, was closer to the quantity he reported. Strain, sex of rat, seasonal variations, or technique may explain this discrepancy.

Addition of the crude protein fraction from human urine to the incubation media of rat adrenal glands *in vitro* promotes an increase in aldosterone secretion. This increment is greater than that of the "corticosterone" fraction. Addition of ACTH causes a different pattern of secretion, namely, a marked rise in the "corticosterone" fraction with a moderate increase in aldosterone.

Four possible explanations of this effect are: (a) It is a non-specific effect. (b) Necessary nutrients, missing from the artificial media, are supplied with the biological fluids. (c) A minute quantity of ACTH present in the biological fluids is producing the observed results. (d) A factor present in human urine specifically stimulates the secretion of aldosterone.

Experimental results presented in Table 4 are evidence against a non-specific effect. Also, addition of the urine protein or serum does not change the potassium to sodium ratio of the media, nor does dialysis destroy the activity of the urine protein. However, failure to establish a dose-response curve is compatible with a non-specific effect.

Presence of nutrients in the biological fluids which are necessary for proper metabolism of adrenal glands *in vitro* cannot be ruled out. Extensive studies by Schonbaum *et al.* (9) found no substance that significantly increased the basal secretion or the response to ACTH of rat adrenals when added to the basic medium (Krebs-Ringer bicarbonate buffer with added glucose). Nevertheless, characterization of such nutrients, if they exist, still would be of biological significance.

In a single *in vitro* experiment by Giroud, *et al.* (1) doses of 1 and 100 milliunits of commercial ACTH preparation stimulated the secretion of aldosterone to the same extent. The 1 milliunit dose has no effect upon secretion of total Δ^4, 3-ketosteroids. Measurement of total Δ^4, 3-ketosteroids, however, includes a number of compounds which are increased little, if at all, by ACTH. Therefore, a small increment in corticosterone secretion might not be detectable. In one experiment by us, a quantity of a highly purified ACTH preparation sufficient to cause a 75% increase in secretion of the "B" fraction had no effect on aldosterone.

If the observed increase in aldosterone secretion is a specific effect of a humoral factor, the possibility arises that this may play a physiological role. Two other lines of evidence lend support to the hypothesis of a humoral factor controlling secretion of aldosterone. Injection of a beef diencephalic extract into dogs has been shown by Farrell *et al.* (10) to stimulate preferentially secretion of aldosterone. Whole urine from sodium-depleted adrenalectomized rats was found by Ralli *et al.* (11) to cause sodium retention in rats with adrenals, but not in adrenalectomized rats.

The present study indicates a humoral factor may influence secretion of aldosterone. Further experiments are in progress to delineate the nature and physiological role of this factor.

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ADRENOCORTICAL INFLUENCES UPON THE RECONSTITUTION OF LYMPHATIC TISSUE FOLLOWING ACUTE INVOLUTION[1]

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ABSTRACT

A single subcutaneous injection of an intoxicating dose of ethyl alcohol, given to intact 17 week old male CBA mice, induced an acute involution of the thymus which was maximal 36 to 48 hours after the injection. Based on a comparison made with the thymo-regressive response to hydrocortisone, the involution observed was estimated to have been due to a fifty-fold increase in adrenocortical steroid activity. A comparison of the growth curves and the cytological mechanisms responsible for the reconstitution of the thymus was then made, between the stressed-intact animals and stressed animals bilaterally adrenalectomized at the time of maximal thymic involution. In the intact animals, the thymus regained 50% of its total weight loss by the 96th hour after the alcohol injections. Beyond this the rate of reconstitution decreased and the normal thymic weight was not attained until between the 240th and 288th hours. Adrenalectomy completely reversed the shape of the growth curve. There was an initial inhibition of growth and a delayed but positively accelerated growth resulting in a hyperplasia of the thymus on the 288th hour. The differences in the growth curves were associated with differences in the cytological mechanisms responsible for growth. It is suggested that there may exist adrenocortical influences which exert stimulating, as well as moderating, effects, upon the reconstitution of the thymus following acute involution.

RECENT investigations concerning the role of the pituitary-adrenal system in acute alcoholism have demonstrated that acute alcohol intoxication induces a depletion of the adrenal ascorbic acid and cholesterol content in intact rats (1, 2) and guinea pigs (3, 4, 5). An acute intoxication induced by the intraperitoneal injection of ethyl alcohol, was also found to induce an involution of the thymus in intact but not in adrenalectomized mice. This phenomenon was then utilized to arrive at a quantitative estimate of the increase in adrenocortical activity associated with the alcohol intoxication. This was done by comparing the thymic involution obtained in intact mice, given two intraperitoneal injections of alcohol, with that obtained in adrenalectomized animals treated with graded doses of hydrocortisone (6).

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The present report deals with studies designed to determine the influences of the adrenal cortical secretions upon the reconstitution of the thymus following the acute involution induced by a single subcutaneous injection of an intoxicating dose of ethyl alcohol. These studies involve the determination of the time required for maximal thymic involution to occur; the estimation of the increase in adrenocortical activity associated therewith; and the study of the reconstitution of the normal mass and cytology of the thymus following the acute involution in intact alcohol-stressed animals. The adrenocortical influences upon the reconstitution of the thymus following acute involution were then determined by comparing the growth curve of the thymus of the alcohol-stressed intact animals with that of alcohol-stressed animals bilaterally adrenalectomized at the time of maximal thymic involution (36 hours post-stress).

EXPERIMENTAL

A total of over 700 male CBA mice 17 weeks of age ± 4 days were used in these studies. These animals were bred in our own colony and were kept under optimal laboratory conditions at all times. Over 30 experiments were performed during a period of 15 months using 20 to 30 animals in each experiment.

The degree and duration of acute involution following alcohol treatment and the time required for the reconstitution of the thymus were first determined. The animals used in this phase of the study are designated as "stressed-intact." As large enough groups of animals came of age, they were treated with a standard intoxicating alcohol dose (6000 mg./kg. of body weight) and groups of at least four animals were sacrificed at various intervals after the injections. At the time of killing, the wet weights of the thymi, peripheral lymph nodes (axillary and inguinal) and paired adrenal glands were determined. These were dissected free of fat and connective tissue and weighed on a Roller-Smith torsion balance accurate to the nearest 0.1 mg. These procedures were repeated until a complete curve of acute involution and reconstitution of the thymus was obtained with at least seven samples from two separate experiments at each time interval following the injection. Initial and final body weights and the losses in body weights were determined for all animals.

The alcohol used was a 50% dilution of absolute ethyl alcohol, U.S.P. and 0.85% pyrogen-free saline. The standard dose of alcohol was volumetrically adjusted, according to the body weight of each animal. This dose, injected subcutaneously, induced an acute intoxication with a resultant mortality of approximately 50% within the first 24 hours. This dose was found to be twice the dosage required to obtain similar intoxicating and lethal effects using intraperitoneal injections as reported in a previous publication (6). The subcutaneous route of administration was used in these studies because it was found that intraperitoneal injections led to a 100% mortality before the thymus returned to normal.

To determine the influence of adrenalectomy upon thymic reconstitution, groups of animals were bilaterally adrenalectomized at the time of maximal thymic involution (36 hours following the alcohol injection); and the subsequent growth curves of lymphatic organs were determined using the standard techniques for the determination of lymphatic organ weights. This group is referred to as "stressed-adrenalectomized."

An estimate of the increase in adrenocortical activity associated with the alcohol-induced stress was obtained by determining the dose of hydrocortisone (Merck) required

to induce a thymic involution in adrenalectomized animals equivalent to the maximal involution observed in stressed-intact animals. This was done using the same techniques as reported in a previous study (6).

Organ weights of all experimental animals were expressed in absolute and relative (mg./100 gm. of initial body weight) terms. The control body and organ weights are the means obtained from intact non-stressed animals sacrificed at the start and termination of each separate experiment. Lymphatic organs were fixed in Zenker-formol and stained routinely with hematoxylin and eosin, and methyl-green pyronin for histological study.

RESULTS

A. *Stressed-intact mice*

Body weight. Following the alcohol injections there was a depression in body weights. This became maximal at 36 and 48 hours post-stress, beyond which the difference between initial and final body weights decreased (Table 1). Beyond the 168th hour post-stress, normal body weight was virtually restored.

Thymus. From a mean control weight of 27.6 ± 1.5 mg. (absolute) and 106.2 ± 5.8 mg. (relative), the thymus lost approximately 63% absolute and relative weight by the 36th hour after injection. Beyond the 48th hour interval there occurred a rapid growth of this organ so that by the 96th hour the thymus regained approximately 50% of the weight loss observed at 48 hours (Fig. 1). Beyond the 96th hour the growth rate gradually decreased and the normal thymic weight was not restored until between the

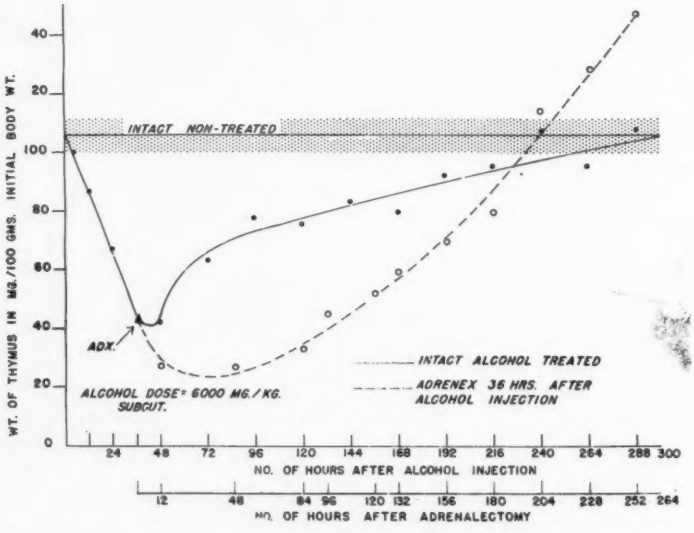

FIG. 1. Curves of involution and reconstitution of the thymus of alcohol-injected CBA mice, intact and adrenalectomized 36 hours after alcohol injection.

TABLE 1. BODY AND ORGAN WEIGHTS OF INTACT MICE AT VARIOUS INTERVALS AFTER A SINGLE ALCOHOL INJECTION, AND FOLLOWING ADRENALECTOMY PERFORMED AT THE THIRTY-SIXTH HOUR AFTER ALCOHOL TREATMENT*

	Hrs. post inj.	Hrs. post adx.	Body weights, gm.†			Organ weights, mg.†					
						Thymus		Lymph nodes		Adrenals	
			Initial	Final	Loss	Absol.	Rel.‡	Absol.	Rel.	Absol.	Rel.
Alcohol injected—Intact	0		24.9± .3			27.6±1.5	106.2± 5.8	10.1± .3	40.4±2.0	2.8±.1	
	4		25.1± .8	24.8± .8	.3± .2	25.2±1.9	99.9± 1.8	9.9± .5	40.2±2.4	3.1±.2	12.5± .7
	12		23.0± .4	22.2± .6	.8± .2	20.0± .9	86.8± 3.1	7.8± .8	34.4±4.4	3.0±.2	12.8± .8
	24		24.9± .8	23.0± .7	1.9± .2	16.9±1.2	67.2± 3.8	7.8± .7	30.4±2.2	2.6±.2	10.7± .8
	36		24.6± .3	22.0± .3	2.6± .2	10.3± .8	43.2± 3.4	9.5± .8	37.5±3.6	3.3±.3	13.9±1.3
	48		25.1±1.0	22.9±1.0	2.2± .2	10.5±1.2	42.2± 4.9	7.8± .8	32.5±3.1	3.6±.4	13.7±1.1
	72		24.9± .6	24.2± .4	1.4± .2	16.8±1.2	63.5± 5.2	9.8± .5	37.7±2.4	2.9±.2	11.0± .6
	96		24.2±1.0	23.5± .8	.7± .2	18.9±1.6	78.3± 6.5	9.2± .7	38.0±2.0	2.6±.2	10.8± .5
	120		24.4± .8	23.7± .7	.7± .1	18.5±1.1	75.8± 4.6	10.6± .5	43.3±1.7	2.9±.1	12.1± .9
	144		25.0± .9	23.9± .9	1.1± .3	20.5±1.0	82.7± 4.0	10.5± .5	41.2±1.9	2.8±.2	10.7± .6
	168		25.3± .5	24.9± .2	.4± .2	20.0±1.3	79.3± 5.3	10.7± .7	43.6±2.5	3.2±.1	12.5± .6
	192		26.0± .6	24.8± .5	1.2± .1	24.0±1.2	92.0± 3.6	17.6±1.3	66.0±3.9	2.8±.1	10.8± .5
	216		26.0± .5	25.1± .6	.9± .2	24.7±1.3	95.0± 4.5	16.0± .6	54.9±2.3	3.0±.3	11.6±1.2
	240		27.4±1.1	26.5± .7	.9± .2	29.5±2.2	106.0± 5.5	16.0±1.5	59.8±8.3	3.1±.2	11.2± .5
	264		28.4± .9	27.9± .9	.5± .2	28.6±2.4	95.0± 4.5	14.5± .3	49.2± .9	2.8±.1	9.7± .3
	288		26.5± .9	26.0± .8	.5± .2	28.2±1.7	106.0± 4.1	19.7±1.5	79.4±5.8	2.6±.3	10.1±1.0
Alcohol injected—Adrenalectomized	36	0	24.6± .3	22.0± .3	2.6± .4	10.3± .8	29.5± 1.5	9.5± .8	37.5±3.6		
	48	12	25.0± .7	22.2± .6	2.8± .4	6.2± .7	27.1± 2.7	4.8± .6	18.8±2.3		
	84	48	28.0± .5	23.8± .5	4.2± .6	7.6± .6	27.7± 2.5	10.8±1.4	39.1±6.3		
	120	84	24.6± .9	21.1± .9	3.5± .4	8.5± .5	33.3± 2.9	7.6± .3	31.7±1.8		
	132	96	24.1±1.2	20.4±1.0	3.7± .6	11.1±2.2	45.1± 7.7	10.4± .6	42.3±5.0		
	156	120	25.5± .2	21.9±2.5	3.6±1.2	11.8±1.1	52.3± 3.2	10.5± .7	41.3±4.2		
	168	132	26.1±1.0	19.6± .3	6.5± .7	10.2±1.2	59.4± 4.9	10.4± .9	38.5±3.7		
	192	156	24.7± .6	21.5± .9	3.2±1.2	17.4±3.8	69.9± 4.4	9.1± .9	36.4±3.6		
	216	180	24.4± .9	20.8± .9	3.6± .6	22.8±3.5	94.5±13.9	11.9±1.9	49.6±8.9		
	240	204	24.2± .5	23.1±1.0	1.1± .9	27.0±4.4	111.9± 9.5	10.1±3.9	34.3±9.9		
	264	228	26.1± .3	22.9± .4	3.2±1.0	33.6±5.3	127.4± 9.9	11.7±1.6	43.9±6.6		
	228	252	27.3± .4	22.8± .7	4.5± .5	37.0±2.0	147.4± 9.8	16.2±1.9	60.6±8.7		

* 6000 mg./kg. body weight, subcutaneously injected.
† Mean and S.E., seven animals/group.
‡ Mg./100 gm. initial body weight.

240th and 288th hour. Histologically it was found that during the initial rapid growth phase (between 48 and 96 hours) there occurred a proliferation of the reticular cells in the thymic cortex (Fig. 2), whereas during the slower growth phase (beyond the 96th hour) there occurred a differentiation of the reticular cells and proliferation of the lymphoid elements, leading toward the reconstitution of the normal cytology of the thymic cortex consisting mostly of small and medium-sized lymphocytes.

Peripheral lymph nodes and adrenal gland. No significant changes were observed in the weights of the peripheral lymph nodes during the first 168 hours following the alcohol stress; however, beyond this point the peripheral lymph nodes underwent a gradual increase in weight so that a significant hyperplasia of these organs was manifest at the 288th hour (Table 1). The adrenal gland failed to undergo significant weight alterations throughout the entire period of observation.

B. *Stressed-adrenalectomized mice*

Body weight. Examination of the changes in body weight in the stressed adrenalectomized animals (Table 1) shows that there occurred an additional loss of body weight by the 48th hour after the operation, at which time there was a loss of $4.2 \pm .6$ gm. Beyond the 48th hour after adrenalectomy the body weight remained 3.5 to 6.5 gm. below normal.

Thymus. Within the first 12 hours following adrenalectomy there occurred an additional loss in the absolute and relative thymic weights (Table 1). The thymus remained small until the 96th hour (132nd hour post-stress). Beyond this hour, there occurred a rapid growth which continued until the thymus of the stressed-adrenalectomized animals was greater than that of the non-stressed controls on the 264th and 288th hour after the injection (Fig. 1). Due to the high mortality we have been unable to pursue our study beyond the 288th hour.

Histological studies revealed that during the latent period (up to the 96th hour post-adrenalectomy) preceding the phase of rapid growth which occurred between the 96th and 252nd hour, there was no cytological evidence of growth. During this period there were a few reticular cells and a greater relative number of lymphoid elements in the thymic cortex (Fig. 3). During the later phase of rapid growth there occurred a proliferation of the residual lymphoid elements but at no time was there observed a preponderant increase in the reticular cells as was observed during the initial period of rapid growth in the stressed-intact animals.

Lymph nodes. Following adrenalectomy there occurred an additional loss in the weight of the peripheral lymph nodes so that within 12 hours after adrenalectomy (48 hours post-stress) the lymph nodes were significantly smaller. However, by the 48th hour after the operation the lymph nodes returned to their normal absolute and relative weights and remained

at normal level for the next 179 hours. A significant increase in lymph node weights was observed at the 288th hour.

C. *Increase in corticoid activity*

The combined results of two experiments designed to obtain an indirect estimate of the increase in adrenocortical activity as induced under the conditions of this study are presented in Figure 4. An estimate of corticoid secretion was made by determining the dose of hydrocortisone required to induce a thymic involution in adrenalectomized animals equivalent to that obtained in intact animals 36 hours after the alcohol injection. In Figure 4,

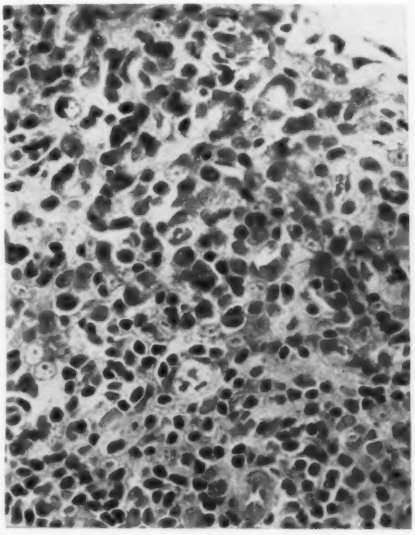

FIG. 2. Thymic cortex of an intact alcohol-treated mouse 72 hours after alcohol injection. Note the large number of normal immature lymphocytes and reticular cells as compared to Figure 3. H. and E. ×500.

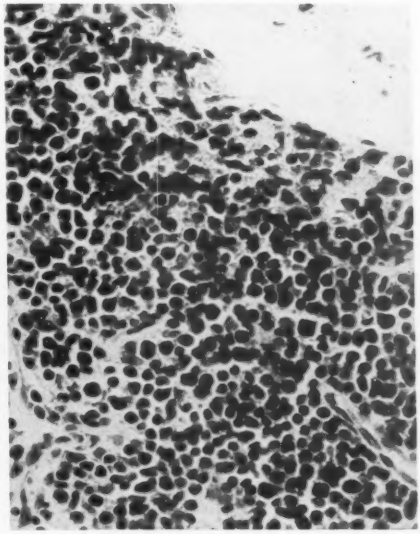

FIG. 3. Thymic cortex of alcohol-treated adrenalectomized mouse, 72 hours after alcohol injection, 36 hours after adrenalectomy. Note the large numbers of small and medium hyperchromatic lymphocytes and few reticular cells as compared to Figure 2. H. and E. ×500.

the relative weights of the thymus are plotted against the log-dose of administered hormone. The horizontal shaded bars represent the mean and standard error of the mean thymic weights of the intact non-treated and of the intact alcohol-treated animals, as obtained 36 hours after the start of the experiment. The sloped line is the thymo-regressive response line mathematically fitted about the observed relative weights obtained from groups of animals which were adrenalectomized, 24 hours later treated with a single injection of one of a graded series of doses of hydrocortisone,

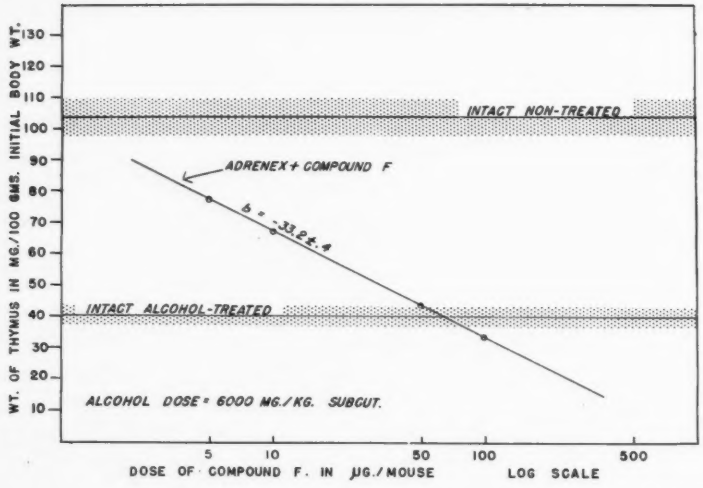

FIG. 4. Log dose-response relationships between the relative weight of the thymus and graded doses of hydrocortisone in adrenalectomized CBA mice. Shaded bands represent mean and S.E. of the thymic weights of intact non-treated and intact alcohol-treated groups.

and sacrificed 36 hours after the hormone treatment. From the intersection of the thymo-regressive response line with the line representing the thymic weights of the control and alcohol-treated animals, it may be seen that a dose of over 50 micrograms of compound F induced a thymic involution equivalent to that induced by the alcohol treatment during an equivalent period of time. This represents about a fifty-fold increase in the hormone dose required to maintain the thymic weight at normal levels in adrenalectomized mice. From this relationship it can be assumed that the subcutaneous alcohol injections in intact animals induced a fifty-fold increase in adrenocortical activity. This agrees with the estimate of increase in corticoid activity arrived at using intraperitoneal injections of alcohol in female mice 10 weeks of age as reported in a previous communication (6).

DISCUSSION

There is an extensive amount of literature concerning the hormonal control of lymphatic tissue (7). For the most part the available information is concerned with those hormones or conditions which favor acute involution, but there is little information concerning the factors which stimulate the growth of lymphatic tissue, either from normal levels or following acute involution. The results presented in this report strongly suggest that there exist adrenal cortical influences which stimulate or enhance the reconstitution of the thymus following acute involution. This is evidenced by the inhibition and alteration in growth of the thymus which occurred

following adrenalectomy performed at the time of maximal stress-induced acute involution.

The mechanisms by which the adrenal cortex may influence the growth of lymphatic tissue following stress are undoubtedly related to the mechanisms involved in the production of acute involution. There are apparently three mechanisms by which the adrenocortical hormones induce acute lymphatic organ involution. They may produce destruction of lymphocytes (lymphocytolysis), and they may also inhibit homoplastic (mitosis) and heteroplastic proliferation (differentiation) of immature cells (7, 8). In a previous study, differential response of lymph nodes and thymus of adrenalectomized mice to the different types of purified adrenocortical products was conclusively demonstrated and mathematically analyzed. It was then suggested that the lymphocytes in different stages of development display different susceptibilities to the lymphocytolytic and mitotic suppressing effects of the C-11 oxy and 17-hydroxycorticosteroids (8). Thus the mature small lymphocytes, which are the cells most characteristic of the thymus, are more susceptible, and the large lymphocytes, characteristically present in relatively greater numbers in lymph nodes, are more resistant to the lymphocytolytic and mitotic suppressing effect of the adrenocortical steroids (7, 8).

Assuming that the stressing effects of the alcohol intoxication, as induced in this study, stimulated the adrenal cortex to secrete steroids of the C-11 oxy and/or 17-hydroxy type, it would then follow that the greatest involutionary effect would be observed in the thymus, due primarily to the lysis of the small lymphocytes. In contrast to the situation in the thymus, the lymph nodes, with relatively larger numbers of reticulo-endothelial cells, would not display a comparable amount of involution and in some cases would display none following acute stress, depending upon the potency of the endogenous adrenocortical secretions.

The mechanism described above would also lead to an understanding of the manner in which the adrenal cortex may influence the thymic reconstitution. At the time of maximal thymic involution (36 to 48 hours after the alcohol injection) the small lymphocytes of the thymic cortex were greatly reduced in number. At this time, however, there was left a well-defined framework of reticular cells and scattered large lymphocytes distributed throughout the thymic cortex. Thus there was a residue of the more resistant lymphocytes and reticular cells. During the initial rapid growth which followed acute involution, there was an increase in the numbers of reticular cells and immature (reticular) lymphocytes. Mitoses among these cells were commonly observed. During the later stages of reconstituion there was a differentiation of reticular cells and proliferation of lymphocytes. It would thus appear that the reconstitution of the normal thymic structure was a reversal of acute involution. Cytological reconstitution appeared in the order of resistance of cells to the effects of steroid hor-

mones, i.e., the most resistant cells were the first to be restored. Reconstitution of the thymus thus would seem to be associated with a progressive decrease in adrenocortical activity.

If the above were correct, it would then follow that the experimental withdrawal of adrenocortical secretions would accelerate the growth of the thymus, but as it was so clearly demonstrated in this study, a withdrawal of adrenocortical steroids, as induced by adrenalectomy, not only failed to accelerate reconstitution, but induced an additional depression in thymic weights and retarded the onset of growth. We are at present unable to account for the additional drop in thymic weight which occurred following adrenalectomy. However, the growth that eventually occurred was extremely rapid and histologically quite unlike the growth which occurred in the intact-stressed animals. The growth of the thymus which occurred in adrenalectomized animals following the alcohol-induced stress was also quite different from that which has been found to occur following adrenalectomy in non-stressed mice of the same strain and age; where a detectable increase in thymic weight and the maximal increment were found at 24 and 120 hours, respectively, following adrenalectomy (9, 11).

The rapid growth that eventually became established after the 120th hour in the stressed-adrenalectomized animals in the present study, was found to be due to an increase in the number of the small lymphocytes of the thymus. Once this homoplastic growth became established, it proceeded virtually unchecked until the thymic weights exceeded those of the normal non-stressed controls. In contrast, homoplastic growth in the intact-stressed animals decreased progressively as the normal thymic weight was approached. The growth mechanisms thus far described suggest that there exists a fine balance of stimulating and moderating influences which serve to control the rate and degree of growth of lymphatic tissue following a stress-induced acute involution.

The involution and reconstitution of lymphatic tissue has also been demonstrated to be related to nutritional factors (7). Comparison of the body weight loss with the thymic involution in the intact animals suggests that the thymic involution which occurred during the initial 48 hours may well have been partly related to the loss in body weight; however, where the maximal loss in body weight was in the order of 9%, the loss in thymic weight was approximately 70%. Thus the body and organ weight loss were disproportionate. Deprivation of the adrenals induced an additional loss in body weight but despite the cachexia which developed, the thymus grew and exceeded its normal size. Thus in the adrenalectomized animals there was growth of the thymus despite the generalized metabolic disturbances.

Administration of the alcohol subcutaneously, instead of intraperitoneally (6), necessitated doubling the alcohol dose in order to obtain as great

an intoxicating and lethal effect. However, on the basis of the thymo-regressive response test, the increase in adrenocortical activity was approximately the same as that obtained using the intraperitoneal route. This comparison might at first seem to imply that the response of the pituitary-adrenal axis to a stressful stimulus would follow an "all or none" law. However, since this work was completed it has been conclusively demonstrated that the adrenocortical response to alcohol-induced stress is proportional to the magnitude of the dose (10). The subjective symptoms of intoxication and the mortality obtained using the subcutaneous route paralleled but were delayed compared with those obtained using the intraperitoneal injections. The need for a larger dose using the subcutaneous route was probably related to a slower rate of absorption. It is at present not possible to determine the manner by which either subcutaneous or intraperitoneal injections of alcohol induce the state of stress. The stress response may conceivably be due to central nervous system effects, tissue damage at the site of injection, or general metabolic disturbances associated with liver damage.

The data and interpretations presented in this study are in close agreement with the theories which have thus far been advanced concerning the manner by which the adrenocortical hormones control the mass and cytology of lymphatic tissue (7, 8). It further appears that the reconstitution of the thymus after acute involution is influenced by adrenocortical factors which exert a stimulating effect upon the reticular cells responsible for a rapid early growth. The adrenal cortex then apparently exerts a moderating effect upon the differentiation of reticular cells and proliferation of lymphoid elements. Deprivation of the controlling factors thus not only inhibits the initial reconstitution but also changes the characteristics of the growth which eventually occurs, leading to a hyperplasia of the thymus and lymph nodes.

Acknowledgment

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STUDIES ON THE ASSAY OF ALDOSTERONE: CHROMATOGRAPHY AND CHEMICAL DETERMINATION[1]

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ABSTRACT

Chloroform extracts from urines were prepared for the determination of aldosterone. These were subjected to various types of paper chromatography and the purified extracts evaluated by the ultra violet absorption at 240 mμ and by the blue tetrazolium color reaction. These results were compared with those obtained by bioassay. A sequence of 4 chromatographic systems was considered necessary for most of the urines to obtain a satisfactory agreement between the chemical and the biological determinations. The chromatographic systems were applied in the following sequence: (1) chloroform-formamide; (2) toluene-ethyl acetate-methanol-water; (3) isooctane-tertiary butanol-water and (4) benzene-methanol-water. With this method, aldosterone was isolated from pregnancy urine and identified by the infra red spectra of the isolated material and its lactone.

The comparison between the results of the bioassay and the chemical determination at various stages of the chromatography indicated that some urine extracts contained, even after separation of hydrocortisone, cortisone and corticosterone from aldosterone, one or possibly more substances which caused a lowering of the results obtained by the bioassay. One such compound appears to be separated from aldosterone by development in the E_2B system.

SOME of the problems connected with the extraction of aldosterone from urines have been described in a previous paper (1). In this communication we wish to present some of our experiences during the past four years with the paper chromatographic separation of aldosterone from crude urinary corticosteroid extracts and its chemical determination. It was considered essential to follow the progression of the chromatographic purification of aldosterone from accompanying steroids by bioassay and to compare these results with values obtained by the chemical evaluation methods. A presentation of such experiments was, however, not possible without mentioning an unknown factor, exerting disturbances in the bioassay, which appears to be present in the urinary steroid extracts, especially from pregnancy urines, even after the removal of cortisone, hydrocortisone and corticosterone. In chromatographic polarity, this factor or

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factors appeared to be closely related to aldosterone. In the bio-assay it would depress the sodium retention in the test animals and on some occasions even lead to frank sodium excretion as compared to the control animals. However, no attempt will be made in this communication to precisely describe such a factor, since further work, which is in progress, is required for its identification.

METHODS

Hydrolysis and Extraction: Urine samples were acidified to pH 1.5 and allowed to stand for one or two days at room temperature over a small volume of chloroform. They were extracted three times with chloroform and the extracts were washed five times with one-tenth volume of 0.1N sodium hydroxide and twice with water. The alkali washings were back extracted with chloroform and the combined extracts evaporated to dryness under reduced pressure.

Paper Chromatography: Aldosterone was isolated from the crude corticosteroid residues by various sequences of chromatographic developments on paper as described later.

(a) General Conditions of Chromatography: The chromatographic tanks were kept in a constant temperature room at 78° F. Whatman paper No. 1 was used throughout since it was found to give the least difficulties with background color. The paper, cut in strips 17 cm. in width, was placed in a Soxhlet apparatus and extracted with methanol for 48 hours. A further extraction with chloroform for an additional 48 hours was carried out on the strips to be used for the final run. After application and chromatographic development the position of hydrocortisone, cortisone and aldosterone on the paper strips was determined by their absorption of ultra violet light. The desired zones were outlined and cut at 1 to 2 cm. above and below the absorbing area. The paper was cut into small pieces which were allowed to stand for 3 hours in methanol or ethanol. The eluate was then filtered through a glass fritted filter. To obtain quantitative elution, the paper pieces were shaken with 3 more portions of alcohol and the combined eluates were evaporated under reduced pressure. Methanol and ethanol eluted the steroids equally well, whereas chloroform and methylene dichloride eluted only 13 and 22% of the applied corticosteroid with the same technique.

(b) Chromatographic Systems: The systems of Zaffaroni (2), using toluene-propylene-glycol, benzene-formamide and chloroform-formamide (3), the latter in the modification by Neher and Wettstein (4), were used for the initial runs. These systems were able to accomplish a good separation of the various corticosteroids from relatively large amounts of the crude steroid extract.

To achieve further separation three more systems were used: they were, the system C of Bush (5) (toluene-ethyl acetate-methanol-water 9:1:5:5), the system B_5 of Bush (5) (benzene-methanol-water 14:6:5) and the system E_2B of Eberlein and Bongiovanni (6) (isocotane-tertiary butanol-water 10:5:9).

When highly pigmented or bulky urinary extracts were obtained, a preliminary purification was carried out before the extracts were processed through the separating systems. This was achieved in the n-heptanol-water system of Schmidt and Staudinger (7) using descending paper chromatography instead of an ascending system as originally outlined by these authors. It was found necessary to introduce a beaker with 300 ml. heptanol into the tank, which was filled with 1000 ml. of water, saturated with heptanol, and to prolong the time allowed for equilibration to 3 hours. Applications were made on Whatman Paper No. 1, previously washed with distilled water and dried. The best results were obtained when a crude neutral residue, equivalent to a 15-hour volume of urine, was applied on a paper 17 cm. in width. Twenty-five ml. water satu-

TABLE 1. CONDITIONS AND MIGRATION RATES IN VARIOUS CHROMATOGRAPHIC SYSTEMS

Chromatographic system	TP	CF	E_2B	BuC	BuB_5	HW	BF
Impregnation of paper	propylene-glycol-methanol 1:1	formamide acetone 5:1	—	—	—	—	for-mamide
Equilibration Time (hrs.)	0	0	5	¾	¾	3	0
Amount of Mobile Phase Used for Development (ml.)	350	25	40	25	25	25	350
Duration of Development (hrs.)	48–72	3.5–4*	18	4–4.5*	4–4.5*	5	5.5
Migration Rate†							
Hydrocortisone (F)	0.470	0.364	0.896	0.632	0.560	1.000	—
Cortisone (E)	1.000	1.000	1.000	1.000	1.000	1.000	0.325
Aldosterone (Al)	0.973	1.010	0.625	0.637	0.857	1.160	—
Aldosterone (mono-acetate)	—	1.600	1.820	1.675	2.075	—	—
Cortisone (mono-acetate)	—	—	—	—	—	—	0.811
11-dehydrocorti-costerone (A)	—	—	—	—	—	—	1.000
11-dehydrocorti-costerone (mono-acetate)	—	—	—	—	—	—	3.400
Aldosterone (diace-tate)	—	—	—	—	—	—	3.360

* Sudan red was added to the standards and papers removed from the tank when the dye had reached the end of the papers.

† With reference to cortisone or 11-dehydrocorticosterone.

rated with heptanol served as the running phase. In contrast with the systems mentioned above, in which an organic solvent is used for the development and in which the urinary pigment and lipid substances are therefore washed over the papers to the solvent front, these materials remain at the starting line in the heptanol water system. In this way they can easily be separated from the corticosteroids which migrate down the paper. Elutates of material remaining at the starting line were repeatedly assayed for a possible occlusion of aldosterone, but none was found under the described conditions. This method has been found to be more advantageous than silica gel columns or petroleum ether-70% alcohol partitions for the purification of urinary extracts.

Details about the development and the running rates of standard corticosteroids in the various chromatographic systems are listed in Table 1.[2]

Physical and Chemical Determinations: The ultra violet absorption of the ethanolic

[2] For convenience the following abbreviations will be used in the tables and in the text:

Chromatographic Systems:

TP—toluene-propylene glycol system.
BF—benzene-formamide system.
CF—chloroform-formamide system.
BuC—system C of Bush.
BuB_5—system B_5 of Bush.
HW—heptanol-water system of Schmidt and Staudinger.

eluate of aldosterone was measured against the eluate from a corresponding strip of blank paper over the range from 200–300 mμ in a Beckman DU Spectrophotometer. The amount of aldosterone present was calculated from the optical density of the absorption maximum:

$$\mu\text{g. steroid/ml.} = \frac{\text{OD max.} \times \text{M} \times 1000}{\text{E}}$$

M = molecular weight (360.4 for aldosterone).
E = molar extinction coefficient (15800 for aldosterone).
λ max. = 238–239 mμ for aldosterone.

The blue tetrazolium (BT) color reaction was performed according to the procedure of Novaczynski *et al.* (8). The reaction was carried out at room temperature and an absorption maximum of the color developed with the BT brand[3] applied was found at 525 mμ. Therefore, this wave length was used for the measurement of the samples instead of 510 mμ. The factors (1/K) for the slopes of the standard curves obtained with cortisone, hydrocortisone and aldosterone were 44.9, 52.2 and 51.6 respectively. With each chemical assay, a standard curve using 5, 10 and 15 μg. of hydrocortisone was dedetermined, against which the samples were evaluated, as the curves for aldosterone and hydrocortisone were found to be almost identical.

For further identification the material was acetylated by the method of Burton *et al.* (2) and the spectra of the sulfuric acid chromogens were determined (9).

Biological Assay: The sodium retaining activity of the urinary extracts was determined by bioassay (10) on adrenalectomized rats receiving a load of 3.5 mg. sodium chloride per rat. One or two groups of animals were injected with desoxycorticosterone acetate (DCA) while the others received urine extracts (in aliquots varying from 10–65 minute volumes per rat). The sodium excretion during the following 4 hours was determined by flame photometry and that of the test animals compared to those of the control and DCA injected animals. For analysis of variance the standard error of the sodium excretion for each animal group was determined. This is listed in Tables 8 and 9 only. In the tables, which present comparisons of results obtained by bioassay and chemical determination in the same patient, the μg. of aldosterone excreted per day are listed with the actual limit of error. These limits of error have been calculated from the standard error of the sodium excretion of each group of test animals and converted into terms of aldosterone.

Standard curves obtained with DCA have shown an index of precision varying from λ = .227 − .260. A standard curve obtained with aldosterone shows an activity approximately 25-fold that of desoxycorticosterone acetate in this assay procedure.

The urinary extracts injected into the test animals for the following experiments were previously purified by various chromatographic procedures as described later.

EXPERIMENTS AND RESULTS

The following experiments were carried out to determine the recovery of aldosterone by bioassay and by the chemical procedure after running the extract in various paper chromatographic systems.

A. Sequence of Chromatographic Systems

Urines collected from a healthy pregnant woman in the ante- and postpartum periods were used in this investigation. They were acidified and

[3] Dajac Laboratories, 5000 Longdon Street, Philadelphia 24, Pa.

extracted as described above. Each of the crude neutral extracts was purified by chromatography in the heptanol water system (15 hours urine aliquot per 17 cm. width of paper). The entire paper, with the exception of the pigmented material along the starting line, was eluted and the steroid residue rechromatographed on two sheets in the TP system for $3\frac{1}{2}$ days. The cortisone zone was eluted and reapplied on one sheet for development in the system BuC for 8 hours. A 10-hour aliquot of the eluate from the hydrocortisone region was submitted to bioassay, while the remaining 20-hour aliquot was further chromatographed on strips 3 cm. wide in system E_2B for 20 hours. The aldosterone zone was eluted and UV-absorption and the BT-color reaction performed on a 10-hour aliquot. The remaining 10-hour aliquot was used for a final development in the system BuB_5 for 7 hours on strips 2 cm. wide. UV-absorption and BT-color reaction were repeated after this step on the aldosterone eluate. The results of this experiment are shown in Table 2.

TABLE 2. ALDOSTERONE ESTIMATIONS AT VARIOUS STAGES OF CHROMATOGRAPHIC PURIFICATION

Normal pregnancy Delivered Sept. 5/57	Aldosterone in μg. per 24 hours				
	(1) After BuC before E_2B Bio-assay (limits of error)	(2) After E_2B before BuB_5		(3) After BuB_5	
		UV	BT	UV	BT
Aug. 15–17	12.1 (7.1–17.3)	164	120	125	115
Aug. 18–20	14.2 (9.4–22.2)	235	133	139	135
Aug. 21–23	37.6 (31.6–44.9)	225	147	145	140
Aug. 24–26	42.7 (33.5–54.6)	254	156	165	150
Aug. 27–29	55.2 (45.0–70.9)	266	178	166	169
Sept. 8–10—Postpartum	16.4 (10.9–24.3)	154	28	18	18
Sept. 11–13—Postpartum	10.1 (6.1–17.3)	101	19	11	11

After the samples were chromatographed in system E_2B the determinations with the UV-absorption method gave considerably higher values for the aldosterone fraction than the BT-color reaction (Table 2, column 2), thus indicating that the material was still contaminated in spite of the previous 4 chromatographic separations. During the last chromatography in system BuB_5, however, this contamination (a UV-positive, BT-negative substance, running a little slower than aldosterone in BuB_5 and probably identical with Genest's Compound V) (19) was separated so that at this stage the ultra violet and blue tetrazolium values agreed fairly well (Table 2, column 3) and the measured compound could be considered as being essentially aldosterone. This was further substantiated by the spectrum of the sulfuric acid chromogen of this material, which showed the characteristic peak for aldosterone at 289 mμ. After heating the sulfuric acid solution to 90° C for one hour, maxima at 247 and at 285 mμ were observed as previously described for aldosterone by Wettstein *et al.* (11). The results which were obtained by means of the bioassay before chromatography in

system E_2B were grossly at variance with the final results from the chemical estimation during the final period of gestation and only the two postpartum samples showed agreement in the three methods. The lower values obtained by bioassay in this patient suggest that some factor is excreted during pregnancy which appears to interfere with the bioassay of aldosterone. This factor has the same chromatographic behavior as aldosterone in the systems TP and BuC and could be separated from it by systems E_2B and BuB_5. This factor was not present in the two urines collected 10 and 13 days postpartum.

B. Separation of Material Disturbing the Bioassay

Extracts from two urine samples collected from healthy women during their sixth month of pregnancy were chromatographed in systems CF, BuC, E_2B and aliquots for bioassay were removed from the crude extract and after each step of chromatography. Chemical analysis was performed

TABLE 3. SEPARATION OF BIOASSAY HINDERING MATERIAL

	Fraction	μg. Aldosterone per 24 hrs.		
		UV	BT	Bioassay (limits of error)
Normal Pregnancy case B.M.	crude extract	—	—	8.6 (5.9–14.1)
	CF—eluate	—	—	10.4 (9.3–12.2)
	BuC—eluate	—	—	—
	E_2B—eluate	24.2	24.5	24.7 (15.6–38.0)
Normal Pregnancy case E.S.	crude extract	—	—	37.1 (29.4–44.9)
	CF—eluate	—	—	—
	BuC—eluate	—	—	31.8 (22.8–43.2)
	E_2B—eluate	45.0	45.6	46.6 (37.5–58.8)

after chromatography in system E_2B. Since agreement between the chemical and biological assays was already reached at this stage, no further development in system BuB_5 was performed (Table 3). The results of the bioassay on the crude extract and the purified fractions from the CF- and BuC paper chromatograms were considerably lower than those obtained after the separation in system E_2B, thus indicating again the presence of a material disturbing the bioassay in the eluates from the CF and BuC chromatograms as well as in the crude extract. In both these specimens the bioassay showed a satisfactory agreement with the chemical determinations after chromatography in the E_2B system. In this respect they behaved differently from the samples in the first experiment (Table 2) which required a subsequent chromatography in system BuB_5 to bring agreement between the UV and BT methods.

C. Background Color from Blank Paper

Since the eluates from blank paper strips showed a certain amount of background color, it was necessary to choose a chromatographic system

for the last step of the procedure which would lead to the smallest blank. Paper strips were therefore subjected to various types of chromatography and color reactions on eluates were carried out with blue tetrazolium and with dinitrophenyl-hydrazine. The results, which are shown in Table 4, demonstrate that the system BuB_5 exhibited the lowest blank values with regard to the blue tetrazolium reaction, whereas system E_2B probably is preferable for the dinitrophenyl hydrazine reaction. The same amount of background color was present in extracts whether they were chromatographed in the 4 systems described or only in the last one. From 24 paper blanks handled during the past six months, the optical density of the background color has varied from 0.030 to 0.050 per 8 cm.^{2} paper, when system BuB_5 was used as the last step.

TABLE 4. BACKGROUND COLORS DEVELOPED FROM BLANK PAPER ELUATES

Chromatographic system	OD of background color from 8 cm.^{2} of paper	
	BT	DNPH
HW	0.035	—
C-F	—	0.067
T-P	0.064	—
BuC	0.096	0.060
E_2B	0.272	0.040
E_2B followed by BuC	0.112	—
BuB_5	0.032	0.056
CF→BuC→E_2B→BuB_5	0.032	—

D. Recovery of Cortisone, Hydrocortisone and Aldosterone

Since a sequence of 4 chromatographic developments was considered necessary for sufficient purification of the aldosterone fraction in the majority of urines examined, it was important to measure the recovery of the steroids applied on the paper after passing through each system. This was determined by UV absorption and reduction of BT. An average recovery of 95.1% was obtained for cortisone or hydrocortisone when chromatographed in amounts of 75–90 μg. in each of the described systems. This would amount to a total loss of approximately 20% after 4 chromatographic developments. In two experiments, when 60 μg. cortisone were chromatographed consecutively in the 4 systems, CF, BuC, E_2B and BuB_5, recoveries of 81.0 and 84.2% of the initial material were observed with the BT- and 83.8 and 85.9 by the UV-evaluation.

When 24.3, 35.2 and 40.0 μg. aldosterone were applied and chromatographed through these four systems, 75.2–77.9% were recovered as evaluated by the UV and BT method, showing an actual loss of about 25% during the chromatographic procedure.

When the free alcohol of aldosterone was added to water, acidified to pH 1.5, allowed to stand for 48 hours at room temperature and extracted

with chloroform in a manner similar to that used for urine, 88% was recovered with the UV- and 85% with the BT-method after chromatography in the system BuC. This demonstrates a loss of about 15% for the hydrolysis, extraction and washing procedure of the aldosterone determination method, including one chromatographic step, so that a total loss of approximately 35–40% would occur during the determination. A similar recovery of 60.8% was obtained when 40 μg. aldosterone were added to the urine before its acidification.

When aldosterone acetate was added to urine samples before acidification, the ester was not quantitatively hydrolyzed under the applied conditions, since only 47.0, 46.5 and 52.8% were recovered after chromatography.

E. Comparison of Aldosterone Values Obtained by Chemical and Biological Assays in Patients with Various Clinical Conditions

Since the four systems, CF, BuC, E_2B and BuB_5 had proven satisfactory when used consecutively for the separation of aldosterone, this combination of chromatographic developments was applied to a series of urines. The eluates from the last chromatogram were subjected to the UV absorption, the BT-color reaction and the bioassay.

TABLE 5. COMPARISON OF ALDOSTERONE LEVELS OBTAINED BY BIOASSAY AND CHEMICAL ESTIMATION AFTER EXTENSIVE CHROMATOGRAPHY

Clinical condition	Aldosterone—μg. per 24 hours		
	UV	BT	Bioassay (limits of error)
Normal males:			
P.S.	2.3	1.8	1.3 (0.9– 1.9)
J.L.	2.6	1.4	2.4 (1.3– 4.0)
M.L.	7.6	7.4	—
J.F.	—	2.1	1.9 (0. – 3.3)
J.F. on ACTH	—	10.9	9.7 (6.4–15.5)
R.M.	—	9.1	8.1 (4.5–14.4)
R.M. on ACTH	—	16.8	12.4 (11.0–14.8)
Normal females:			
I.R.	4.7	1.1	1.2 (0 – 4.9)
O.R.	9.4	7.0	8.1 (6.3–11.2)
D.M.	2.0	1.9	2.2 (1.4– 3.8)
S.A.	4.8	4.2	5.9 (4.2– 7.9)
L.G.	—	5.1	6.7 (3.3–11.2)
L.D.	—	3.8	2.2 (0 – 4.0)
I.P. (1)	—	3.1	3.4 (1.9– 5.9)
I.P. (2)	—	2.4	3.2 (2.1– 4.9)
I.P. (3)	—	3.9	4.2 (2.9– 6.9)
Hypokalemia—J.B.	12.6	14.3	13.2 (11.1–16.1)
Cushing syndrome—L.L.			
untreated	17.0	11.4	12.2 (9.2–17.1)
on ACTH	24.9	24.8	22.2 (19.0–27.1)
post ACTH	8.6	8.5	8.9 (6.6–12.9)
post adrenalectomy	neg.	<1.0	<1.0
Sodium loosing syndrome—R.G.	neg.	<1.0	<1.0
Cushing's syndrome—M.C. untreated	—	5.9	6.3 (4.1– 9.8)

Table 5 shows the results obtained in healthy persons, two patients with Cushing's disease, one case of hypokalemia and a case with a sodium losing condition. In general, fairly good agreement occurred between the chemical methods and the bioassay. In four instances higher levels were obtained by measurement of UV absorption indicating that this determination was the one most sensitive to contaminations.

Table 6 shows aldosterone values obtained during a metabolic study on a case of panhypopituitarism receiving growth hormone preparations. The BT color reaction generally agreed well with the bioassay whereas here again the results of the UV absorption measurements were not so reliable. In the instances indicated by dashes in Table 6, no satisfactory UV absorption curve was obtained. The clinical conditions of this patient were discussed as "case 2" by Beck *et al.* (36).

F. Isolation of Aldosterone from Pregnancy Urine

Thirty-six liters of urine obtained from healthy women in their last month of pregnancy were extracted in the manner described under "Methods.' For purification from pigments the total crude steroid residue was chromatographed in the heptanol-water system using 16 sheets of paper. The brown starting line region was excluded and the rest of the papers eluted. This material was then applied and developed on eight sheets of paper in system CF. From this chromatogram the dark band absorbing light in the region of cortisone, was eluted including 3–4 cm. above its upper borderline, thus excluding the urinary hydrocortisone. A chromatography in system BuC followed using 8 paper strips, most of which showed 4 UV-light absorbing zones. The band in the region of hydrocortisone

TABLE 6. COMPARISON OF ALDOSTERONE LEVELS OBTAINED BY BIOASSAY AND CHEMICAL ESTIMATION AFTER EXTENSIVE CHROMATOGRAPHY IN A CASE OF PANHYPOPITUITARISM (G. L. AGE 18)

Day of	Treatment	Aldosterone—μg. per 24 hours		
		UV	BT	Bioassay (limits of error)
1– 3		3.8	2.7	2.9 (1.9– 4.3)
4– 6		7.2	6.8	7.6 (5.9–10.2)
7– 9		8.1	7.9	7.0 (4.5–10.4)
11–12	Porcine Growth Hormone 10 mg.	6.5	5.5	3.7 (2.7– 5.6)
13–15	Porcine Growth Hormone 40 mg.	4.4	5.0	2.6 (1.6– 4.3)
16–18		2.3	2.7	2.7 (1.8– 4.4)
19–21		6.1	—	5.9 (4.5– 7.2)
22–24		—	—	4.5 (2.3– 8.6)
25–27	Human Growth Hormone 2 mg.	—	10.9	9.6 (7.3–12.1)
28–30	Human Growth Hormone 2 mg.	—	14.0	14.6 (11.9–18.4)
31–33	Human Growth Hormone 5 mg.	(8.0)*	(7.2)*	17.8 (11.3–27.9)
34–36	Human Growth Hormone 10 mg.	9.6	10.2	10.8 (6.9–16.7)
37–39	Human Growth Hormone 20 mg.	15.0	14.1	13.6 (6.8–16.4)
40–42		8.2	7.3	8.0 (5.1–14.7)
43–45		—	6.0	5.3 (4.0– 7.2)
46–48		—	6.5	7.3 (4.5–16.3)
49–51		4.2	4.7	4.0 (3.4– 4.8)

* Some of this material was lost during the procedure.

standard was eluted for development in system E_2B on the same number of papers. Three zones were observed, the center one corresponded to the position of aldosterone. This was eluted and rechromatographed in system BuB_5, which did not separate any visible amounts of UV light absorbing compounds (Genest's Cpd. V) and in this behaved differently from the urine used in experiment A (Table 2) but similar to the two pregnancy urines in experiment B which also showed no evidence for the presence of Cpd. V. The aldosterone region showed a material which absorbed UV light maximally at 239 mμ. The amounts of this material were estimated by the three available methods and are shown in Table 7. The results determined by bioassay for aldosterone activity, increased with progressive purification and reached the levels measured by the BT-reaction after the last chromatography, thus indicating again, for the crude fractions, an inhibition of the aldosterone effect in the test animals by some contaminating material, some of which was removed by development in the E_2B-system,

TABLE 7. ALDOSTERONE ISOLATED FROM PREGNANCY URINE

System	Aldosterone in μg.		
	UV	BT	Bio-assay (limits of error)
After BuC	790	3660	328 (267–396)
After E_2B	518	760	414 (337–521)
After BuB_5	478	480	468 (397–569)

whereas a remainder was separated out by BuB_5. An infra red spectrum of the isolated material was obtained through the kindness of Dr. Lieberman and Dr. Ulick. We are also indebted to them for the preparation of the lactone 874 of Reichstein (12) from our material and for the infrared spectrum of the latter substance. Both spectra were considered compatible with the ones of authentic aldosterone and of lactone 874 respectively. When the lactone prepared from the urinary material was chromatographed in cyclo-hexane-benzene (1:1)/formamide with the authentic compound for $6\frac{1}{2}$ hours, both showed the same running rate (lactones 19.25 cm., adrenosterone 22.25 cm.).

An acetate was prepared from material isolated in a similar manner from other batches of pregnancy urine. This substance showed, in system BF, the running rate expected for aldosterone diacetate (Table 1). The sulfuric acid chromogen spectrum had a single maximum at 289 mμ and exhibited peaks at 247 and 285 after heating to 90° C, which added further evidence for the identity of the isolated material with aldosterone.

DISCUSSION

The necessity for a purification of the crude neutral corticosteroid extracts had already been recognized at the time when only bioassay methods were available for the determination of aldosterone. Hydrocortisone, corti-

sone, corticosterone, which are known to occur in these extracts, may affect the excretion of sodium and/or potassium in adrenalectomized animals. In our assay procedure cortisone was found to lower the aldosterone results when present in amounts of 25 μg. or more per rat; 1–10 μg. had little effect on the results (Table 8). Hydrocortisone, when injected directly into test animals, showed a sodium excreting effect at dose levels from 30 to 50 μg. per rat, whereas lower levels did not appear to have any significant influence. Corticosterone also produced some sodium excretion at levels of 5–15 μg. per rat, but did not alter the sodium excretion at 20 μg. per rat. Luetscher and Johnson (13) found that hydrocortisone, cortisone, corticosterone, as well as 11-dehydrocorticosterone, had an excreting effect in their assay procedure and therefore chromatographed the crude urinary extracts in

TABLE 8. INFLUENCE OF ADDED CORTISONE ON THE ALDOSTERONE RESULTS OF THE BIO-ASSAY ON A PREGNANCY URINE

Urine sample Min. vol./rat	Cortisone added μg./rat	Na-excretion Meq. with standard error	Aldosterone μg./24 hrs.
10	0	.139± .016	7.00
10	1	.140± .012	6.85
10	5	.141± .013	6.60
10	10	.141± .008	6.60
10	25	.172± .015	3.10

the TP and/or the BuB_5 system before submitting them to bioassay. The observations of various authors with regard to the sodium retaining or sodium excreting activity of cortisone and hydrocortisone at various dose levels in their respective assay methods, have been summarized by Streeten *et al.* (14).

The purification of the aldosterone fraction from crude extracts is even more of a necessity when the hormone is measured chemically, since the available evaluation methods are group reactions, the formazan color measuring the α-ketol group in the sidechain, the ultra violet absorption the Δ^4-3-keto group in ring A, and the formation of dinitrophenyl hydrazones the keto groups in general. Only the latter reaction may, according to Gornall and Gwilliam (15), be considered at least partially specific for aldosterone, since conditions can be chosen which lead to an absorption maximum of 460 mμ for the aldosterone derivative, while most other steroids show, with the same treatment, maxima at 495 mμ.

Simpson and Tait (16) were the first to demonstrate an effective separation of aldosterone from cortisone and hydrocortisone by the use of the two paper chromatographic systems TP and BuC. Subsequently, Neher and Wettstein (17), developed a physico-chemical method for the measurement of aldosterone in urine, based upon these procedures. A preliminary purification of the chloroform extract with a partition between petroleum

ether and aqueous ethanol, followed by a column chromatography on silica gel, were carried out. The eluates from the BuC chromatograms were evaluated by a semi quantitative method, which compares the intensity of the BT-color and soda fluorescence of the aldosterone fraction with hydrocortisone. In the following year the authors reported an improvement in their procedure (4) and replaced the TP by the CF system, which required only 4 hours running time instead of 3 days. They also mention in this article the possibility that in such eluates, which are free of cortisone, hydrocortisone and corticosterone, other factors might occur.

More recently, Novaczynski, Genest *et al.* (18, 19) showed that other compounds actually were present in the aldosterone region of the BuC chromatogram and that more than two chromatographic developments were needed for a complete separation of aldosterone from these substances. So far they have characterized six compounds, in addition to aldosterone, from urines of patients with Cushing's disease and from that of healthy pregnant women. They reported that compounds III and V occur most frequently. Compound III was found to be increased in patients on high intakes of potassium (19) and compound II is believed to be the 11-keto analogue of aldosterone. To separate all these compounds completely from aldosterone the authors suggested, after purification of the extracts on a silica-gel column, the use of at least 3 chromatographic systems in the following sequence: ethylene-glycol-toluene, E_2B and BuB_5.

Gornall and coworkers (15, 20, 21) also suggested that more than two chromatograms are needed to obtain a pure aldosterone fraction and proposed a sequence of two modified BuB_5 systems (addition of 5 and 10% ethylacetate to the benzene) and the TP system.

Hernando and coworkers (22) observed that the eluate of the aldosterone zone, obtained after purification in 3 systems, the florisil column and the TP and BuC systems of paper chromatography, divided into 3 zones when run in the E_2B system. These were aldosterone, compounds III and /or IV of Genest and another compound more polar which reduced BT. In our own experiments, we have observed compounds III or IV separating from aldosterone in the E_2B system in most of the urines as a UV light absorbing, BT not reducing material, traveling a little faster than cortisone. Compound V was observed from the pregnancy urine of experiment A, when it was separated by the BuB_5 system. An additional compound (BT positive and UV negative), not described by Novaczynski *et al.* (18,19) was observed to be separated in the final BuB_5 system from the pregnancy urine of experiment F (Table 7). After its removal the bioassay showed higher results.

These various observations indicate that there are several compounds present in urine with running rates on paper similar to aldosterone. These do not occur consistently in all urines and are often present in only trace

amounts. To a great extent these are derived from the dark absorbing zone seen directly below the cortisone region on the initial papers run in the CF system. This zone is excluded when the Neher and Wettstein method is followed precisely. In practice, however, and when the TP system is applied, the cortisone zone and this zone often overlap and it is difficult to obtain a clear-cut separation; therefore, it is felt to be safer to include the upper part of this dark zone and rely on the subsequent systems for a more complete separation of aldosterone.

Another sequence of chromatographic separations has been suggested by Moolenaar (23, 24) who used toluene-octanol-methanol and water in two systems with different component ratios to separate aldosterone. A modified method of Gornall was used for the final evaluation.

All these assays are sensitive to approximately 1 μg. of aldosterone. The recovery of added aldosterone from urine varies as follows, Neher and Wettstein (4) 75%; Novaczynski *et al.* (19) 83%; and Hernando (22) 20–31%. Our recovery is around 60%.

Lately more sensitive methods have been developed with the help of isotopes, which will allow aldosterone determinations in blood. Ayres and coworkers (25), using column chromatography on Kieselgur were able to measure aldosterone diacetate-C_{14} in the range from 0.2 to 4.0 μg. with great precision. Kliman and Peterson (26), labeling the hormone with tritiated acetate, reported a sensitivity of 1/100 μg. of aldosterone.

When the results of the chemical assay were compared with those of the bioassay, a generally good agreement was observed when the samples were extensively purified (Tables 3, 5 and 6). Lower values were often obtained by bioassay from crude extracts and from eluates after preliminary chromatography, than from the chemical determination as shown in Tables 2 and 3. Such differences may be primarily due to the interfering action of hydrocortisone, cortisone or corticosterone for all assays done on the crude extracts. Differences remaining after chromatography in the systems CF and BuC must, however, be interpreted as a contamination of the aldosterone fraction with other substances, either actively sodium excreting or aldosterone antagonizing, which have running rates similar to this hormone in the TP, CF and BuC systems (see Experiments, A, B and F). Tables 3 and 7 indicate that one of such materials can be separated in the E_2B system; whether this is identical with Compound III of Novaczynski *et al.* (35) is not established at the present time. Another one seems to require the BuB_5 system (Table 7).

The possibility of the existence of material with such biological effects was suggested to us by several observations during our experience with the bioassay in the past three years. An extract, roughly purified in the BuB_5 system for the purpose of removing compounds F, B, and most of Compound E, was frequently found to show less aldosterone when assayed at a

higher dose level than at a lower dose level (Table 9). That this effect could not be explained on the basis of traces of cortisone which had not been completely removed is evident from the data presented in Table 8. The biological action of the material affecting the bioassay of aldosterone exerted its influence only in higher concentration, and could be diluted out to a certain degree. Other evidence for the presence of sodium excreting substances was observed in certain clinical conditions characterized by a sudden active output of sodium in the patient. The extract of such urines, when injected into adrenalectomized animals, actually increased the sodium output above that of the control group. This effect was observed in a patient with liver disease at a time when the ascites was spontaneously

TABLE 9. EXAMPLES OF URINE EXTRACTS, PURIFIED BY BuB_5 CHROMATOGRAPHY, WHICH SHOW A GREATER INTERFERENCE IN THE BIOASSAY ON HIGHER DOSE LEVELS OF INJECTION

Case	Clinical Condition	Aldosterone by Bioassay in μg./24 hours*			
		10 min.†	15 min.†	30 min.†	45 min.†
N.L. (1)	Normal Pregnancy	9.1 (.149± .017)‡		138%ex. (.265± .023)‡	
(2)		15.6 (.123± .022)		3.9 (.136± .015)	
(3)		26.8 (.098± .015)		9.8 (.094± .017)	
J.S.	Normal Pregnancy		5.1 (.173± .013)		132%ex. (.297± .027)
S.B. (1)	Toxemia of Pregnancy		16.3 (.114± .021)		7.3 (.098± .009)
(2)			26.5 (.089± .014)		10.0 (.082± .013)
(3)			39.1 (.068± .010)		11.5 (.075± .016)
(4)			57.6 (.047± .008)		14.4 (.061± .006)
K.M. (1)	Toxemia of Pregnancy	13.1 (.100± .014)§		7.2 (.082± .012)§	
(2)		15.2 (.094± .011)		3.0 (.113± .015)	
(3)		34.0 (.066± .008)		6.8 (.084± .007)	
J.K. (1)	Toxemia of Pregnancy	9.1 (.134± .008)		3.1 (.133± .016)	
(2)		13.4 (.118± .007)		3.3 (.131± .011)	
G.Z.	Toxemia of Pregnancy	24.5 (.110± .017)		12.5 (.088± .019)	
G.J.	Toxemia of Pregnancy	10.3 (.156± .021)		3.3 (.159± .022)	

* The figures listed in parentheses indicate the sodium excretion of the test animals in m.eq. with the standard error.
† Minute volume injected per rat.
‡ Sodium excretion of the control group: .193± .024 m.eq.
§ Sodium excretion of the control group: .225± .019 m.eq.

being resorbed and sodium diuresis was occurring (27). Occasionally this effect is observed after ACTH withdrawal in normal individuals or in patients when the injected extract caused a significant excretion of sodium in the test animal. Certain urines from pregnant women also appear to contain such a factor (Table 9).

Similar observations have been mentioned by Neher and Wettstein (4) who obtained more crystalline aldosterone from the urines of patients with nephrosis and with cardiac failure than the biosassy had previously indicated. Since this material which is affecting the bioassay is chromatographically so closely related to aldosterone, one might speculate that it could be derived from the hormone during the procedure, possibly by oxidation. In this connection it is interesting to note that recently Wettstein (28) reported on the sodium excreting properties of Reichstein's compound No. 885 (34), which is the lactone of an aldosterone analogue carrying a carboxyl group in the C_{18} position.

The identification of the urinary sodium retaining material with aldosterone was first established by Luetscher, Neher and coworkers in neph-

rosis (29, 30) and later in cardiac failure (31). Aldosterone was also chemically identified in the urine of patients undergoing surgery (32) and in the urine of pregnant women by Novacznyski *et al.* (18). In our own studies the characterization from pregnancy urine is further substantiated by the infra red spectrum and acetate formation of the hormone obtained from pregnancy urine.

The assay of aldosterone either by chemical or biological procedures is still a time-consuming operation. Only a small fraction of the amount of hormone secreted by the gland appears in the urine as free steroid. The recent report of Ulick and Lieberman (33) of the isolation of a tetra-hydro derivative of aldosterone present in much larger quantities than aldosterone in urine offers another means of studying the metabolism of this hormone in clinical conditions.

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EFFECTS OF ACUTE AND CHRONIC TREATMENTS WITH CARBUTAMIDE (BZ-55) ON OBESE-HYPERGLYCEMIC MICE AND THEIR LEAN LITTERMATES[1]

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ABSTRACT

The acute administration of carbutamide was found to have a hypoglycemic effect in fasted normal mice, while it had no definite effect on the already low blood sugar levels of fasted obese hyperglycemic mice. Chronic oral treatment with carbutamide did not modify the food intake and weight increases of either type of mice during the seven weeks treatment. In obese-hyperglycemic mice no improvement of the "diabetes" was observed, both in fed and fasted states. Lean littermates, whether fasted or fed, showed a reversal of the direction of effect of a single dose. These animals were, rather paradoxically, rendered paradiabetic after four weeks of treatment, and responded to glucagon and growth hormone with a reaction similar to that seen in untreated obese-hyperglycemic mice.

THE hypoglycemic action of carbutamide (BZ-55) and related sulfonylureas is said to take place only in the presence of insulin. Several hypotheses have been advanced to explain this effect: carbutamide may promote increased insulin secretion or it may potentiate the action of insulin, this in turn either at the hepatic level or/and in extrahepatic tissues (1, 2). It has also been suggested that sulfonylureas act by decreasing hepatic phosphorylase activity (3, 4). The availability of mice with the hereditary obese-hyperglycemic syndrome seemed to offer an opportunity to elaborate the mechanism of action of carbutamide. These animals, besides showing obesity and a relatively insulin resistant type of hyperglycemia (5) exhibit an increase in the insulin content of their pancreas (6) and an increased hepatic phosphorylase activity (7). Obese-hyperglycemic mice seem therefore particularly suitable for the testing of hypothesis relating the effect of carbutamide to the potentiation of insulin or to decrease in hepatic phosphorylase activity. It appeared useful to use carbutamide both in acute and in chronic treatments on both obese-hyperglycemic mice and their normal littermates.

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MATERIAL AND METHODS

25 obese-hyperglycemic and 27 littermates were kept in individual cages on ground Purina chow. As nearly as possible equal numbers of males and females were used in each group. As the duration and development of the hyperglycemic may influence the response to carbutamide, mice of 2 age ranges were distributed among the groups in the proportion of 2 young (2 months old) to 1 adult (4 months old) (see Table 1).

TABLE 1. CHRONIC TREATMENT WITH CARBUTAMIDE

Average daily food intake and final weight increase (in percentage from the starting values) of obese-hyperglycemic mice and their lean littermates. Comparison between control animals and animals treated orally with carbutamide.

	Untreated			Treated with carbutamide		
	No.	Average daily food intake, gm.	Weight increase	No.	Average daily food intake, gm.	Weight increase
Littermates:						
Young	9	5.3	16	9	4.9	14
Adult	4	5.1	0	5	6.0	5
Total	13	5.2±1.4		14	5.2±1.4	
Obese:						
Young	9	6.3	35	9	5.9	32
Adult	4	6.5	16	3	6.6	9
Total	13	6.4±1.4		12	6.1±1.2	

A. Chronic experiment

After a control period of 3 weeks, 12 obese and 14 littermates received carbutamide uniformly mixed with the ground chow (2.5 gm. carbutamide per Kg. of diet) while the remaining (control) animals were maintained on unmodified diet. During the period of 10 weeks throughout the experiment, the following records and determinations were made:

1. the food intake, once a week (corrected for spilling, which occurred to a larger extent in lean littermates than in the obese)
2. the postprandial weight of each mouse, one to three times a week
3. the postprandial blood sugar on days 1, 28, 35, 42, 49, and 70 (approximately at 10 A.M. every day)
4. the fasting blood sugar after 17 hours on days 2, 36, and 56.

In addition, the effects of growth hormone and HGF were studied in the following manner:

Bovine growth hormone (Armour list 916, potency 106%, contamination with ACTH less than 6×10^{-5} U.S.P./mg., with TSH less than 8×10^{-3} U.S.P./mg. and Prolactin less than 10^{-1} I.U./mg.) was dissolved in distilled water, and 1 mg. injected subcutaneously on the 42nd day. The blood sugars were checked immediately before and 17 hours after the injection, on fed animals.

HGF (crystalline Lilly CT 871, with an insulin content of less than 5.10^{-2} U./mg.) was dissolved in a phenol solution (pH 2.5) to a final strength of 40 μg./ml. 10μg. were injected intraperitoneally in each fed mouse on the 49th day. The blood sugars were determined immediately before and 20 minutes after the injection.

B. Acute experiment

The acute effect of carbutamide was studied on the same control and treated animals fasted for 17 hours, on the 56th day of the chronic experiment. The carbutamide was

dissolved in a NaOH solution, pH 8.0 and 12.5 mg. of the sulfonylurea injected subcutaneously into each mouse. The blood sugar was determined before and one hour after the injection.

Blood was collected from the tail vein and determinations were carried out on 0.05 ml. samples by the method of Somogyi-Nelson (8, 9) (filter 66 for low readings and filter 54 for high readings). Prior to the sampling, the mice were lightly anesthetized (10 mg. nembutal/100 gm. body weight).

RESULTS

A. Chronic experiment

No trend in intake during the course of the experiment having been discovered, the data in each group of mice were pooled and compared (Table 1). Carbutamide, when mixed with the food did not modify the food intake of obese mice and their littermates, whether young or adult. The relative hyperphagia of obese-hyperglycemic mice (6.4 gm. daily instead of 5.2 gm. for the littermates in this series) persisted. The weight increases are graphically recorded in Figure 1 and expressed in percentage from the starting values in Table 1. Again there was no difference between treated and untreated animals. Young obese hyperglycemic mice gain

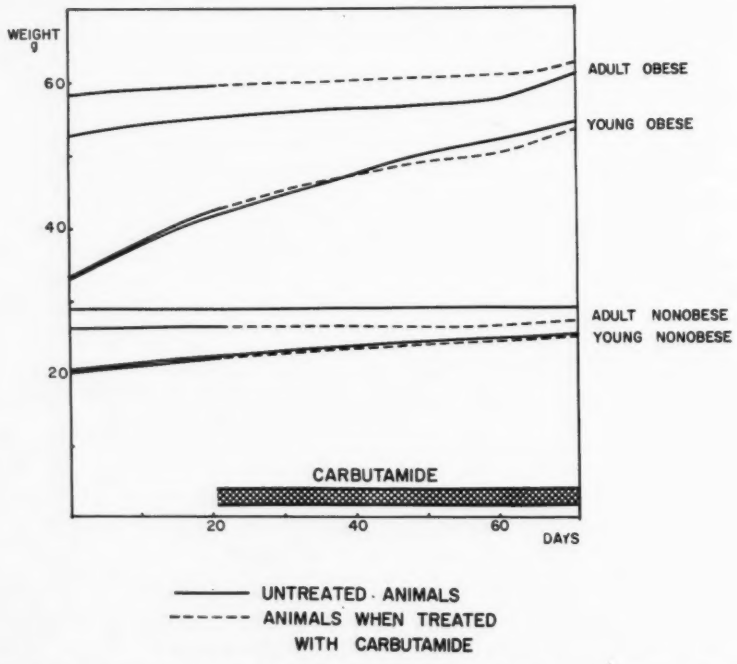

FIG. 1. Average weight curves of adult and young obese-hyperglycemic mice and their non-obese littermates. After a control period of 3 weeks, half of the animals in each group received carbutamide mixed with the chow (2.5 gm. per Kg. of diet) while the remaining mice were maintained on the unmodified diet.

TABLE 2. EFFECT OF A CHRONIC TREATMENT WITH CARBUTAMIDE ON THE BLOOD SUGAR LEVELS OF OBESE-HYPERGLYCEMIC MICE AND THEIR LEAN LITTERMATES

(Values are given in mg. per 100 ml.)

	Dietary status	Day of experiment	Blood glucose of untreated young and adult mice			Day of treatment‡	Blood glucose of carbutamide-treated young and adult mice		
			Young (9)*	Adult (4)	Young and adult†		Young (9)	Adult (5)	Young and adult†
Littermates	Fed	1	108	86	101 ± 23	—	98	98	98 ± 21
		28	90	104	94 ± 16	7	109	113	110 ± 29
		35	125	131	127 ± 19	14	152	139	147 ± 42
		42	108	113	112 ± 24	21	130	124	128 ± 19
		49	94	95	94 ± 21	28	146	141	152 ± 51^{xx}
		70	89	89	89 ± 21	49	112	133	120 ± 31^{xx}
	Fasted (17 hrs.)	2	41	58	46 ± 39	—	42	53	46 ± 33
		36	53	82	62 ± 35	15	85	75	81 ± 27
		56	64	55	62 ± 21	35	92	84	89 ± 23^{xx}
	Dietary status	Day of experiment	Blood glucose of untreated young and adult mice			Day of treatment	Blood glucose of carbutamide-treated young and adult mice		
			Young (9)	Adult (4)	Young and adult†		Young (9)	Adult (3)	Young and adult†
Obese	Fed	1	212	—	—	—	220	275	237 ± 55
		28	211	243	221 ± 84	7	204	264	219 ± 75
		35	231	195	220 ± 111	14	178	235	192 ± 96
		42	245	192	229 ± 133	21	210	221	213 ± 128
		49	233	180	217 ± 118	28	207	150	197 ± 81
		70	236	193	223 ± 118	49	202	204	203 ± 57
	Fasted (17 hrs.)	2	79	67	75 ± 28	—	92	72	87 ± 51
		36	66	96	75 ± 43	15	112	118	117 ± 51^{x}
		56	25	33	28 ± 32	35	81	115	91 ± 43^{xx}

* Numbers in parentheses are numbers of animals.
† There was no significant difference between the blood sugar levels of young and adult mice. The standard deviations of the average blood levels are therefore only given for the combined data.
‡ The animals receiving carbutamide were treated orally with the drug from the 21st day of the experiment.
^{x} and ^{xx}: differences between treated groups and control groups significant at 5% and 1% levels respectively.

weight rapidly in both treated and untreated groups, while the weight of the littermates increased slowly.

The chronic oral treatment with carbutamide increased the blood sugar level of the lean mice (Table 2). After 28 days of treatment this increase was significant both in the fed and in the fasting states. After the same duration of treatment the hyperglycemia of obese-hyperglycemic mice was not lessened. The slight decrease of postprandial sugar levels was not significant. Moreover, the chronic treatment appeared to decrease the extent of blood sugar fall resulting from fasting.

Growth hormone administered to control animals fed *ad libitum* decreased blood sugar when it was measured 17 hours after administration to mice which did not receive carbutamide (Table 3). This effect was not observed on lean animals who had received carbutamide in their diet for 43 days. In obese-hyperglycemic mice the slight hyperglycemic effect of growth hormone (10) was enhanced by carbutamide treatment. As expected (5) the observed hyperglycemic effect of an intraperitoneal injection of 10 μg. of glucagon was greater in the obese than in the lean group (Table 3). Carbutamide increased the hyperglycemic response. This increase was significant in the lean group, not significant in the obese group.

B. Acute experiment

The subcutaneous administration of 12.5 mg. of carbutamide to fasted

TABLE 3. BLOOD GLUCOSE LEVELS OF OBESE-HYPERGLYCEMIC MICE AND CONTROLS UNTREATED OR CHRONICALLY TREATED WITH CARBUTAMIDE WHEN SUBJECTED TO INJECTION OF GROWTH HORMONE, GLUCAGON AND CARBUTAMIDE

Type of animal and number	Chronic treatment	Acute treatment	Day from start of experiment	Dietary status	Blood glucose, mg. per cent			Effect of treatment on blood glucose and significance	
					Before treatment	After treatment	Change	Difference, mg. per cent	Significance
Littermates (13)	None	Growth Hormone 1 mg. subcutaneously	42	Fed	112 ± 24	74 ± 21	−38 ± 34	44 ± 31	$p < 0.01$
(14)	.25% Carbutamide in diet	Growth Hormone 1 mg. subcutaneously	42	Fed	128 ± 19	134 ± 31	6 ± 28		
(13)	None	Glucagon 10 μg. intraperitoneally	49	Fed	94 ± 21	129 ± 43	35 ± 41	71 ± 52	$p < 0.01$
(14)	.25% Carbutamide in diet	Glucagon 10 μg. intraperitoneally	49	Fed	152 ± 51	257 ± 71	106 ± 62		
(13)	None	Carbutamide 12.5 mg. subcutaneously	56	Fasted	62 ± 21	23 ± 19	−39 ± 25	43 ± 27	$p < 0.01$
(14)	.25% Carbutamide in diet	Carbutamide 12.5 mg. subcutaneously	56	Fasted	89 ± 23	93 ± 32	4 ± 28		
Obese (13)	None	Growth Hormone 1 mg. subcutaneously	42	Fed	229 ± 133	237 ± 110	9 ± 65	16 ± 82	$p < 0.5$
(12)	.25% Carbutamide in diet	Growth Hormone 1 mg. subcutaneously	42	Fed	213 ± 128	236 ± 106	25 ± 100		
(13)	None	Glucagon 10 μg. intraperitoneally	49	Fed	217 ± 118	343 ± 117	126 ± 78	51 ± 66	$p < 0.1$
(12)	.25% Carbutamide in diet	Glucagon 10 μg. intraperitoneally	49	Fed	197 ± 81	374 ± 87	177 ± 47		
(13)	None	Carbutamide 12.5 mg. subcutaneously	56	Fasted	28 ± 32	52 ± 55	24 ± 34	38 ± 36	$p < 0.02$
(12)	.25% Carbutamide	Carbutamide 12.5 mg. subcutaneously	56	Fasted	91 ± 43	77 ± 60	−14 ± 36		

lean mice receiving the drug for the first time, lowered significantly the blood glucose level (Table 3). Animals treated orally for 56 days with carbutamide no longer showed this effect. In obese-hyperglycemic mice, not previously treated with oral carbutamide there was some inconsistent increase in blood glucose after an injection. In chronically treated animals there was practically no modification of blood glucose.

DISCUSSION

The daily consumption of carbutamide during the chronic experiment averaged 0.50 gm./Kg. of body weight in the lean animals, and 0.30 gm./Kg. in the obese. These daily oral doses were approximately equal to those given subcutaneously in the acute experiment. Although these doses are large on a ratio-to-body-weight basis, when compared with the dosages given to human patients, they have no deleterious effects on the appetite or on the weight of young and adult animals.

The *acute administration* of carbutamide was found to be hypoglycemic in fasted lean mice. This is similar to results obtained in normal animals of other species. Nevertheless, it was without effect on fasted obese-hyperglycemic mice. These mice have hypertrophied β cells and a pancreas with a high insulin content (6). Several authors have indicated their belief that insulin is necessary for the action of carbutamide, the evidence presented here demonstrates that presence of this hormone in the pancreas does not necessarily guarantee a successful therapy. However, this observation does not rule out the hypothesis that carbutamide acts to release insulin from the pancreas. Since it has been shown that the hyperglycemia of these mice responds only slightly to moderate doses of exogenous insulin (6), endogenous hypersecretion may also be relatively ineffective. A more likely alternative is that the pancreas of obese-hyperglycemic mice already produces and releases all the insulin it is capable of so that additional stimulation is without effect. The hypoglycemia observed in lean mice after the first single subcutaneous dose of carbutamide is not observed in the group previously subjected to oral treatment with the drug. This suggests that some adaptation to the treatment takes place. Indeed the slight hyperglycemia induced in chronically treated lean mice, whether fasted or fed, shows a reversal of the direction of effect of a single dose. Finally, and rather paradoxically, lean mice on the chronic treatment are rendered paradiabetic, while the condition of the obese-hyperglycemic remain unchanged. Lean mice, chronically treated with carbutamide respond to glucagon and growth hormone with a reaction similar to that seen in untreated obese-hyperglycemic mice (5, 10). Chronic administration of carbutamide enhances the hyperglycemic response to glucagon, a fact perhaps related to increased liver glycogen following treatment with sulfonylureas (11, 12) (but in apparent contradiction with results obtained *in vitro* (3, 4)). Carbutamide treatment also eliminates in these non-obese mice

the hypoglycemia occurring after growth hormone injection.

An increased food consumption is not involved in the development of this paradiabetic condition in normal mice. An enhanced neoglucogenesis seems unlikely since a normal pancreas should be able to control the hyperglycemia due to neoglucogenesis. There remains the possibility of decreased glucose utilization. In order to explain this decreased glucose utilization, one can hypothesize that a pancreatic exhaustion occurs secondary to a period of hyperactivity. An alternative hypothesis is that in mice as in rats (13, 14) and in dogs (15) the insulin sensitivity decreases during the chronic treatment with carbutamide.

Comparison of data obtained here in mice with data obtained in other animal species indicates that chronic treatment with carbutamide has effects which are functions of species, dose and duration of treatment. In fasted dogs, the hypoglycemic effect is still observed and the rate of glucose utilization enhanced after several weeks of treatment (15). In fasted rats, hypoglycemic effect and improved glucose utilization (13) fade concomitantly. After two weeks of treatment, the rate of glucose utilization is decreased (14), this decrease still persists after 3 months (16). Conflicting results have been obtained in studies of oral glucose tolerance in both species subjected to chronic treatment with sulfonylureas (17, 18, 19). It now appears that the mouse shows yet a different type of reaction: in obese-hyperglycemic mice, we observe essentially no improvement of the "diabetic" condition; in normal mice there is a slowly developing tendency toward a paradiabetic condition.

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INFLUENCE OF GONADAL HORMONES ON THE METABOLISM OF CORTISONE

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ABSTRACT

Liver homogenates from adult male rats reduced the 17,21-dihydroxy-20-ketone sidechain of cortisone more rapidly than did similar preparations from female rats. Conversely, the female preparation metabolized the $_{\Delta}4$–3 ketone group at a faster rate than did the male. The ratio A-ring reduction/sidechain reduction for the female is 3.4; for the male it is 0.88. The sex difference in the metabolism of the sidechain of cortisone is not present in rats younger than about 25 days of age but becomes evident by the time the animals are 30 days old. Gonadectomy and injections of estradiol-17β and testosterone indicate that the male hormone promotes the ability of the liver to reduce the sidechain of cortisone while the female hormone has an inhibitory effect.

IN THE course of the metabolism of adrenocortical hormones reduction of the C-20 and $_{\Delta}4$–3 ketone groups is known to occur. These reactions occur in man (1) and in various laboratory animals; can be demonstrated both in the intact animal and in liver slices, homogenates and subcellular fractions (2, 3, 4, 5). Enzymes involved in these conversions have been characterized as to intracellular localization (5), substrate specificity (3, 4), and cofactor requirements (5, 6, 7). The effects of various endocrine factors on the reduction of the A-ring and sidechain of adrenocortical steroids have been studied. Brown and co-workers (8) have reported pretreatment of rats with cortisone or triiodothyronine increases the ability of liver slices to metabolize hydrocortisone while hypophysectomy or thyroidectomy reduces the rate of metabolism. Likewise, Yates et al. (9) and McGuire (10) have found thyroid hormones to increase the rate of reduction in ring A of adrenocortical steroids by liver slices and homogenates. This paper reports effects of gonadal hormones on the enzyme systems of the liver responsible for reduction of the C-20 and $_{\Delta}4$–3 ketone groups of cortisone. A preliminary report on this subject has appeared (11).

METHODS

Rats from the Holtzman strain were used throughout this study. The selected rat was killed by cervical section and exsanguinated. A portion of the liver was rapidly excised, chilled and homogenized in 0.25 *M* sucrose made 0.04 *M* with nicotinamide. Homogenization was performed at 0° C in a Potter homogenizer with a Teflon pestle. Four ml. of whole homogenate containing 200 mg. tissue were incubated with 150 μg of cortisone added in 0.1 ml. ethanol. Incubation was for 2 hours at 37° C in an atmos-

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phere of nitrogen. The pH was maintained at 7.4 with 0.1 ml. of molar potassium phosphate buffer. The TPN system consisted of 0.01 ml. of 0.3 *M* manganese chloride, 0.1 ml. of 0.2 *M* sodium isocitrate and 0.4 ml. of 10^{-4} *M* TPN. The addition of isocitric dehyrogenase was found to be unnecessary for maximal TPN effect. However, it is noted that whole homogenates were used in these experiments and endogenous isocitric dehydrogenase was doubtlessly present in the system.

Following incubation the homogenate was extracted with 20 ml. chloroform. The chloroform layer was washed with 0.1N NaOH and 0.1N HCl and a 1 ml. aliquot evaporated to dryness. Alteration of the sidechain of cortisone was followed by the Porter-Silber reaction (12) after the dry residue had been taken up in 1 ml. of methanol. Reduction of the 3-keto $-_{\Delta}4$ function in ring A was determined by adding 4 ml. of methylene chloride to the dried aliquot and reading the resulting solution in a Beckman DU spectrophotometer at 240 mμ. In each experiment a duplicate, unincubated control flask was prepared, to which chloroform was added immediately after the addition of cortisone. The data were analyzed by the small sample t test and mean differences were considered significant if the value of P was 0.01 or less.

RESULTS

The results presented in Table 1 indicate that liver homogenates from adult male rats reduce the sidechain of cortisone at a faster rate than do the corresponding preparations from female rats. Addition of the TPN system produced an increased rate of cortisone reduction by both male and female preparations. The increase was relatively greater with female liver but the marked sex difference is still apparent. The disappearance rates of the $_{\Delta}4$–3 ketone position of cortisone, incubated in the presence and absence of the TPN system, are also shown in Table 1. In this instance the situation is the reverse of that seen with respect to sidechain metabolism in that the female reduces the A-ring more rapidly than does the male. This is apparent whether or not TPN is added to the system.

Various experiments were performed to elucidate further the nature of the observed sex difference in the metabolism of cortisone. Information on A-ring changes is still incomplete and for this reason only observations on

TABLE 1. THE METABOLISM OF CORTISONE BY LIVER HOMOGENATES AS MEASURED BY THE PORTER-SILBER REACTION AND ULTRA-VIOLET ABSORPTION AT 240 mμ. THE SEX DIFFERENCE AND EFFECT OF TPN ARE SHOWN

	No. of animals	Micrograms of cortisone lost				E 240 mμ/Porter-Silber with added TPN system
		Porter-Silber reaction		E 240 mμ		
		Without added TPN system	With added TPN system	Without added TPN system	With added TPN system	
Normal males (300–350 gm.)	17	72 ± 2	104 ± 5	41 ± 5	92 ± 2	0.88
Normal females (220–240 gm.)	16	12 ± 2	32 ± 2	60 ± 3	109 ± 2	3.4

Results in this and in the following table are given as means ± S.E.

TABLE 2. THE EFFECT OF GONADECTOMY AND OF GONADAL HORMONES ON THE RATE OF REDUCTION OF THE 17-21-DIHYDROXY-20-KETONE SIDECHAIN OF CORTISONE BY LIVER HOMOGENATES. THE TPN SYSTEM WAS NOT ADDED

Group No.		No. of animals	Micrograms cortisone lost
1	Castrated males	6	45 ± 5
2	Estradiol treated males	6	19 ± 2
3	Castrated males treated with estradiol	6	16 ± 2
4	Ovariectomized females	7	25 ± 4
5	Testosterone treated females	6	30 ± 6

side chain reduction are presented in Table 2. The animals represented in this table were similar in age and weight to the normal rats of Table 1 and direct comparisons can be made. The operated male animals were castrated 15 days prior to removal of the liver for study. The females were ovariectomized 20 days before study. Gonadectomy is seen to have a marked effect on both the male and female but in opposite directions, i.e., gonadectomy decreases the rate of cortisone reduction in the male and increases the rate in the female. The male rats in Groups 2 and 3 of Table 2 were treated with estradiol-17β, 60 μg./day subcutaneously for 12 days. Cortisone metabolism is obviously depressed in these preparations.

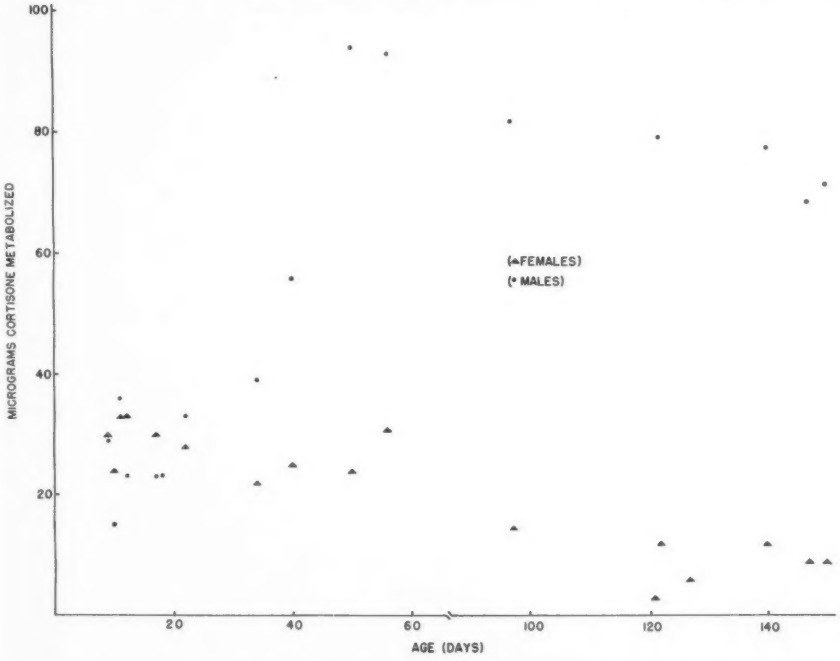

FIG. 1. Influence of age and sex on the metabolism of cortisone by liver homogenates. Cortisone was measured by the Porter-Siber reaction. The TPN system was not added.

Testosterone promotes the ability of liver tissue from female rats to reduce the sidechain of cortisone. This is shown in Group 5 of Table 2. These animals were given 2.5 mg. testosterone propionate per day for 15 days intramuscularly.

It was of interest to determine the age at which male and female rats first begin to metabolize cortisone differently. Figure 1 indicates no difference during the first 20 days of postnatal life. However, by 30 days of age the values for the two sexes are clearly divergent due to a sharp increase in the activity of male liver and a moderate decrease in that of female liver. The male appears to reach a peak value at about 50 to 60 days of age and to gradually decline thereafter.

DISCUSSION

The studies presented indicate that homogenates prepared from the liver of male and female rats metabolize the sidechain and A-ring of cortisone in a quantitatively different manner. The male reduces the sidechain more rapidly than does the female who, in turn, reduces the A-ring at a greater rate than does the male. This is in line with the observation of Yates and coworkers (9) that liver slices from female rats have a greater capacity for reducing the A-ring of cortisone than do slices from male rats. It is also of interest to note that the female rat metabolizes the A-ring to a greater extent than the sidechain (Table 1) while the male more nearly reduces these sites on the molecule to the same extent. This is especially the case in the presence of TPN. The ratio A-ring reduction/sidechain reduction for the female is 3.4; for the male it is 0.88.

The action of triphosphopyridine nucleotide in accelerating the rate of reduction of the sidechain and A-ring (Table 1) is in agreement with much previous work (4,6,7,8). Glenn and coworkers (13) working with the microsome-supernatant fraction of rat liver, have observed that the C-20 ketone group is metabolized faster than the Δ4–3 ketone function of hydrocortisone. However, alterations in substrate and cofactor composition were stated to be capable of reversing this process. Sex of the animals studied was not given and in view of the present findings this may be an important factor.

That the sex difference in the metabolism of the sidechain of cortisone is dependent upon gonadal secretions is clearly indicated by the data in Table 2, the male hormone tending to increase and the female hormone tending to decrease the rate of reduction. These effects are further pointed up in Figure 1 with the marked sex difference becoming apparent at about the time significant endocrine activity of the gonads would be expected.

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THE EFFECT OF ANTERIOR HYPOPHYSEAL ADMINISTRATION ON THE OVARIES OF OLD RATS[1]

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ABSTRACT

Following the removal of the left (control) ovaries, groups of immature, young adult and old rats received homologous anterior lobe implants daily for 10 days; the right (stimulated) ovaries were obtained at autopsy. The ovaries were examined in serial sections for the numbers and sizes of corpora lutea and follicles, and for the presence of "wheel cells" in the interstitial tissue.

The ovaries of the old rats showed a variable response (increase in weight and an increase in the number and size of follicles and of corpora lutea) to anterior lobe implants, but for the group as a whole, there were statistically no significant differences between the "control" and "stimulated" ovaries for the weights of the organs or the numbers of corpora lutea, and only borderline significance for the number of follicles. However, increase in the size of the follicles and corpora lutea in the stimulated ovaries was more pronounced and the differences, as measured by the mean diameter of these structures, showed a high degree of statistical significance. Wheel cells were present in 75% of the "control" ovaries and in only 29% of the "stimulated" ovaries. The reaction of the "stimulated" ovaries of the immature and young adult rats to anterior lobe administration was much more pronounced and relatively more constant than that which occurred in the old rats.

THE primary cause of ovarian regression which is associated with aging has not been definitely established, although waning anterior hypophyseal activity and a decreased capacity of the ovary to respond to stimulation by the anterior lobe have both been suggested. In old anestrous mice it has been found that a single implant of anterior lobe tissue induced an increase in weight of the ovaries accompanied by a growth of follicles, formation of corpora lutea and estrus (1, 2, 3). Following the administration of either anterior lobe tissue or urinary gonadotrophin, Weisner (4) noted essentially the same reaction in aged rats and expressed the view that the regression of the gonads was due to the lack of a specific stimulating factor. In contrast, in studies in which women of various ages received either pregnant mare serum (PMS) or an extract of castrate urine, Watson, Smith and Kurzrok (5) concluded that the causal factors in ovarian regression were inherent in the ovary itself and that this organ became refractive to gonadotrophic stimulation, a view also held by Masters (6).

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More recently Ortiz (7) found, correlated with increasing age, a small and gradual decrease of the response of the ovaries of hamsters to PMS, although the ovaries of old animals were still quite reactive. Finally, in mice ranging in age from 3 to 19 months, Green (8) reported that PMS induced a pronounced and fairly uniform increment in ovarian weight up to the age of 15 months; in increasingly old animals the response progressively decreased.

In studies (9, 10) pertinent to the present investigation it was found that, correlated with advancing age, the ovaries of rats were characterized by a progressive decrease in the number of normal follicles and corpora lutea, an increase in follicular atresia, and evidence of a partial failure of ovulation. In many animals, "wheel" or "deficiency" cells similar to those first described in the ovaries of hypophysectomized rats (11) appeared. Both a deficiency of anterior lobe stimulation and the incapacity of the ovary to respond to such stimulation have been suggested as causal in the appearance of these cells in old rats (9, 12). The present communication is concerned with the comparative responses of the ovaries of relatively old and relatively young female rats to anterior lobe administration.

MATERIALS AND METHODS

Female rats between the ages of 22 and 32 months and groups of immature (approximately 30 days old) and young adult rats (approximately 90 days old) received anterior lobe implants. Routinely, the left (control) ovary of each animal was removed; the animals then received 10 daily intramuscular implants of a fresh anterior lobe from a normal adult male rat. The animals were killed 48 hours after receiving the last implant, and the right (stimulated) ovary and the remainder of the reproductive tract were removed. All tissues were fixed in Bouin's fluid, embedded in paraffin, and stained with hematoxylin and eosin. Serial sections of the ovaries and representative sections of uteri and vaginae were studied.

The ovaries were examined by the quantitative methods described by Wolfe (9). The follicles and corpora lutea in each ovary were counted; their sizes were determined by taking the average of their two greatest diameters. Only follicles with a mean diameter of at least 0.3 mm. and corpora lutea with an average diameter of at least 0.5 mm. were included in the counts; follicles with an average diameter of 0.9 mm. or more were regarded as cystic.

In the ovaries of old rats, where relatively small numbers of follicles were present, the normal, atretic, and cystic follicles were counted separately; unless specifically noted, however, they will be considered below as a single group. In the younger rats, the stimulated ovaries contained so many follicles that it was impractical to count the three types separately. Since it was not feasible to measure the sizes of all the follicles and corpora lutea in the young animals, random sampling was used, in each case adequate in number to assure a statistically reliable average.

RESULTS

A statistical analysis of the over-all differences between the various responses to anterior lobe stimulation, such as weights, number and size of follicles and corpora lutea, of the control and stimulated ovaries of the old

TABLE 1. THE EFFECTS OF IMPLANTATION OF ANTERIOR LOBES ON OVARIES OF OLD RATS

Number of Rats = 28

	Control	Stimulated	Standard error (SD)	Mean 1−Mean 2 / SD
Mean weights of ovaries (mg.)	36 ± 4	44 ± 3	5.7	1.4
Mean number of follicles per ovary	9 ± .9	12 ± 1	1.4	2.1
Mean diameter of follicles (mm.)	.55 ± .03	.72 ± .01	.03	5.0
Ovaries with cystic follicles	43%	89%	—	—
Ovaries with corpora lutea	70%	89%	—	—
Mean number of corpora lutea per ovary	6 ± .7	8 ± 1	1.2	1.8
Mean diameter of corpora lutea (mm.)	.91 ± .05	1.13 ± .04	.05	4.4
Ovaries with direct luteinization	19%	56%	—	—
Ovaries with "wheel" cells	75%	29%	—	—

rats and of the younger animals is presented in Tables 1 and 2. The last column of these tables gives the significance ratio; a ratio between 2 and 2.5 is about 95% significant. Certain of these responses, or quantitative differences in the responses between the control and stimulated ovaries of individual rats, are indicated in Figures 1 and 2.

Reference to Table 1 will indicate that while in the old rats the mean weight of the stimulated ovaries was increased, the degree of the increase was not statistically significant. Furthermore, the responses among the individual rats were quite variable; in 19 rats the stimulated ovaries were heavier to varying degrees than the controls, but in 7 instances the stimulated ovaries actually weighed less than the controls, and in two rats there was no change (Fig. 1).

The mean number of follicles per ovary was slightly and barely significantly increased in the stimulated ovaries (Table 1), but again examination of data from individual rats indicates that the responses varied widely (Fig. 1). In direct contrast, the average size of the follicles was pronounc-

TABLE 2. THE EFFECTS OF IMPLANTATION OF ANTERIOR LOBE TISSUE ON THE OVARIES OF IMMATURE AND YOUNG ADULT RATS

	Immature rats No. = 9				Young adult rats No. = 8			
	Control Ovary	Stimulated Ovary	Standard error	$M_1 - M_2$* / SD	Control Ovary	Stimulated Ovary	SD*	$M_1 - M_2$* / SD
Mean weights of ovaries (mg.)	14 ± .6	106 ± 14	14	6.5	49 ± 2	168 ± 24	24	5
Mean number of follicles per ovary	17 ± 1.7	52 ± 10	10	3.5	29 ± 3.9	71 ± 15.6	15.8	2.7
Mean diameter of follicles (mm.)	.39 ± .02	.60 ± .02	.02	10.5	.46 ± .03	.69 ± .05	.05	4.6
Ovaries with cystic follicles	0%	67%	—	—	0%	100%	—	—
Ovaries with corpora lutea	0%	100%	—	—	100%	100%	—	—
Mean number of corpora lutea per ovary	0	17 ± 2.8	—	—	15 ± 1.4	21 ± 3.2	4.2	1.4
Mean diameter of corpora lutea (mm.)	0	1.18 ± .06	—	—	.88 ± .04	1.26 ± .06	.07	5.4
Ovaries with direct luteinization	0%	67%	—	—	0%	50%	—	—

* M = mean. SD = Standard error.

edly increased after anterior lobe administration, the increase being highly significant (Table 1). Cystic follicles also occurred much more frequently in the stimulated ovaries (Table 1). However, the increased size of the follicles in the stimulated ovaries was not entirely due to the increased occurrence of cystic follicles, for when the normal follicles were considered alone, it was found that they were significantly increased in mean diameter.

The mean number of corpora lutea in the stimulated ovaries was slightly

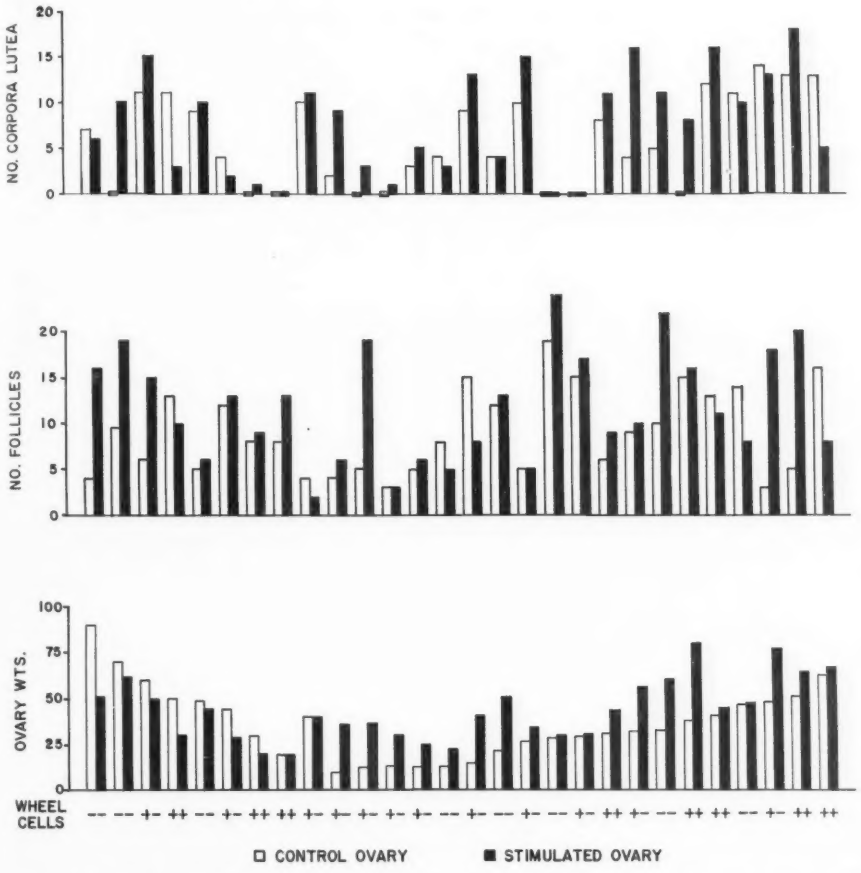

FIG. 1. The number of corpora lutea, the number of follicles and the weights, in mg., of the control and stimulated ovaries of the old rats are indicated by the three rows of bars which are arranged so that these three responses from a single animal are directly in line, one above the other. Control ovaries are represented by open bars, stimulated ovaries by solid bars. The very low bars cutting the base line in the first row indicate that no measurable corpora lutea were present. Also indicated at the bottom of the figure are the presence or absence of wheel cells in the individual ovaries.

greater than that in the controls, but the increase was statistically insignificant (Table 1), and it will be noted in Figure 1 that an increase actually occurred in only 16 rats; in the remainder there was either a decrease or no change. However, as in the case of the follicles, the corpora lutea were found in stimulated ovaries to be on the average significantly larger than those of the controls (Table 1). In many ovaries, histological examination revealed that numerous corpora lutea had formed by direct luteinization, as entrapped ova were found; the number of ovaries containing corpora lutea formed in this fashion was markedly increased in the stimulated ovaries (Table 1).

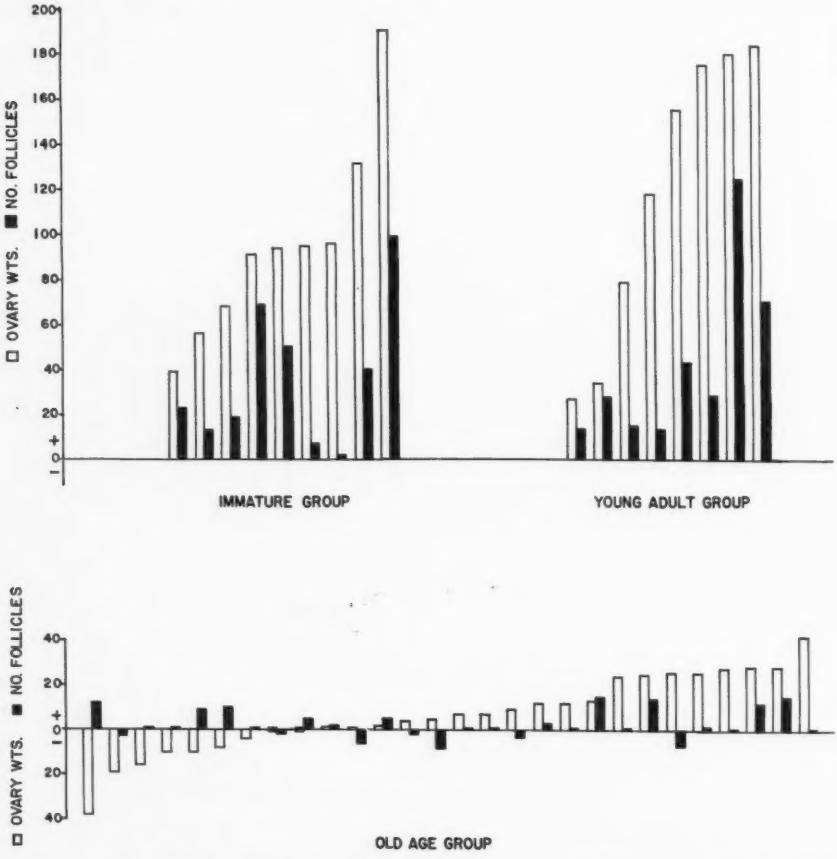

FIG. 2. The heights of the pairs of bars represent actual differences in the weights in milligrams (open bars) and in the number of follicles (solid bars) between the control and stimulated ovaries of individual rats. Negative differences (i.e., when a control ovary was heavier than the stimulated) lie below the base line, positive differences above. The very low bars cutting the base line indicate that no differences occurred.

Seventy-five per cent of the control ovaries contained wheel cells in the interstitial tissue, while such cells occurred in only 29% of the stimulated ovaries (Table 1). The absence or the presence of these cells in the control and stimulated ovaries of individual rats is indicated in Figure 1.

A statistical analysis of the over-all response of the ovaries of the immature and young adult rats to anterior lobe treatment is presented in Table 2. In general, the reaction was much more pronounced and much less variable than that in the old rats. The stimulated ovaries of both groups of the younger rats were significantly heavier than the control ovaries (Table 2). In Figure 2, where the difference between the weights of the control and stimulated ovaries of each rat is recorded, both the increased response of the younger rats and the lesser variability are quite clear.

The numbers of follicles were significantly increased in the stimulated ovaries of both groups of younger rats (Table 2). While the degree of such increase varied among the individual rats, the consistency of increased production of follicles in every ovary stimulated by anterior lobe tissue is evident from the fact that no columns are seen below the zero line (Fig. 2, upper row). Furthermore, the over-all response greatly exceeded that which occurred in the old animals, where the increase in the mean number of follicles was of only slight significance (Table 1) and actually occurred in only 19 of the 28 stimulated ovaries (Fig. 1).

The mean diameter of the follicles in the stimulated ovaries of both groups of young rats was significantly increased (Table 2), and there was a pronounced increase in the numbers of cystic follicles (Table 2), reactions which also occurred in the stimulated ovaries of the old rats.

In the young adult rats, it was found that the mean number of corpora lutea was not significantly increased after anterior lobe administration but that significant increase in size did occur (Table 2). In addition, increased numbers of luteinized follicles were found in the stimulated ovaries. In these responses, the old and the young adult animals responded similarly (compare Table 1 with Table 2).

The question arises whether there were consistent correlations between the various responses to anterior lobe administration in the individual rats. In both the immature and young adult rats such correlations were fairly consistent. As an example, it is shown in Figure 2 that the increase in weight of the individual stimulated ovary was invariably accompanied by an increase in the number of follicles in the same ovary. In the immature rats the increase in ovarian weight was accompanied by an increase in the number of corpora lutea; in the young adult rats, however, an increase in the number of corpora lutea failed to occur in three of the stimulated ovaries. In the ovaries of the old rats, there was no consistency among the various responses. An example of this finding is presented. Among the 19 old rats where the stimulated ovary was larger than the control, there was

an increase in the number of follicles in 12 instances (Figs. 1, 2), disappearance of wheel cells in 9, and an increase in number of corpora lutea in 12 (Fig. 1). However, of the 9 rats in which the stimulated ovary either was no heavier or actually weighed less than the control, there was also an increase in the number of follicles in 7 rats (Figs. 1, 2), disappearance of wheel cells in 3, and an increase in the number of corpora lutea in 5 rats (Fig. 1).

In the above analysis the weight increase or lack of weight increase was used as a point of reference with which the other responses were compared. In a further analysis of the data presented in Figure 1, an increase or lack of increase in the number of follicles, an increase or lack of increase in the number of corpora lutea, and the disappearance or persistence of wheel cells in the stimulated ovaries were successively used as the base of reference. Again, a similar lack of consistency between the various responses was noted in all of these comparisons.

Testis-like tubules seemingly identical to those described by Engle and others (13, 14, 15, 16) were present in the ovaries of all of the old rats. Although not subjected to quantitative analysis, the tubules appeared to be equally abundant in the control and stimulated ovaries; thus, there was no evidence to indicate that they were affected by the anterior lobe administration. These tubules were not found in the immature animals, but a few occurred in the ovaries (both control and stimulated) of three of the young adult rats. The present findings, therefore, suggest that their appearance in the ovary of the rat is a manifestation of advancing age; this relationship is at present receiving further attention.

In the region of the hilum, the ovaries of 4 of the old rats contained large papillary intracystic proliferations resembling the papillary cystadenomata illustrated by Moon and his associates (15). Whether these are structural changes related to aging is open to further consideration.

DISCUSSION

The fact that the ovaries of most old rats are capable of responding to the administration of anterior lobe tissue would support the view that ovarian regression in aged rats is due to reduced activity of the anterior lobe. However, the lesser intensity of the response as compared to that found in younger rats, and the pronounced variability of the response, strongly suggest that the development of a refractory state in the ovary is an important factor in the decrease and in the final termination of gonadal activity in old female rats. The degree of response to stimulation shown by the ovaries of old rats varies within wide limits.

Unpublished studies which have been carried out in this laboratory have a bearing on this problem. Ovaries were exchanged between pairs of rats, one young and one old, by reciprocal transplantation into the kidneys. It was found, on the basis of observations on the estrous cycle and subsequent

histologic examination of the transplant, that the transplantation of "young" ovaries in old rats was much more successful than the transplantation of "old" ovaries in young hosts. Thus, the factors determining the survival and function of the graft seemed to be predominantly inherent in the ovary itself. It should be noted, however, that Li and Gardner (17) have found that the ovaries of old mice developed tumors when transplanted into the spleen or the pancreas of young animals, and that they seemed to be just as capable of forming tumors under these conditions as did the ovaries of young mice. However, these two sets of experiments are not comparable, since the latter authors were determining the capacity of the ovary for atypical growth rather than its capacity for maintenance of normal structure and function.

Although the present studies strongly suggest that the ovaries of old rats become less responsive to stimulation by the anterior hypophysis, it is possible that lessened ovarian reactivity is not the only factor involved. Thus it has been reported (9) that correlated with advancing age in the female rat there is evidence of a decrease of secretory activity in the anterior lobe cells, and certainly, since ovarian and anterior lobe activity are so intimately linked, it is not unreasonable to assume that aging changes in the anterior lobe are concerned in ovarian regression. In her appraisal of this problem in hamsters, Ortiz (7) concludes that reproductive involution is not due to a complete loss of function on the part of the anterior lobe or to a refractive state of the ovary but rather to a decrease in the functional activities of many organs leading to an endocrine imbalance.

SUMMARY

The ovaries of aged, immature and young adult rats, before and after administration of homologous anterior lobe implants, were studied in serial sections.

The ovaries of the old rats responded to anterior lobe implants, but the degree of the response was reduced and the response was much more variable than that of the ovaries of younger rats.

The "stimulated" ovaries of the old rats were not significantly increased in weight, nor were the numbers of corpora lutea significantly increased. An increase in the numbers of follicles occurred, which was barely significant. In contrast, the mean size of the follicles was pronouncedly and significantly increased. The corpora lutea likewise were significantly increased in size. The ovaries of the immature and young adult rats responded more intensely to anterior lobe treatment. The increase which occurred in the weights of the ovaries and the increases in the number and size of the follicles were statistically significant. In the young adult group, the corpora lutea were significantly increased in size but not in number.

"Wheel" cells were present in 75% of the control ovaries of the old rats,

but were found in only 29% of the stimulated ovaries. Anterior lobe administration induced the formation of many cystic follicles and pronounced direct luteinization in the ovaries of both the young and old rats.

Comparison of the various categories of response by the stimulated ovary of each rat demonstrated a relatively consistent positive correlation in the immature and young adult rats, i.e., a positive response in one category was generally accompanied by other structural evidences of stimulation. Such positive correlations of responses were not evident in the old rats.

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THYROID HORMONE ADMINISTRATION AND OVARIAN AND ADRENAL ACTIVITY IN RATS BEARING HYPOTHALAMIC LESIONS[1]

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ABSTRACT

Bilaterally symmetrical electrolytic lesions which damaged arcuate nucleus and median eminence in the hypothalamus of adult female rats regularly produced atrophy of the reproductive tract (and reduced thyroid function) but were without consistent effect on adrenal morphology. Hypothalamic obesity and genital hypoplasia were frequently associated. Thyroxine and triiodothyronine administration to intact animals induced ovarian and uterine hypertrophy as well as adrenal enlargement. Similar treatment in operated rats failed to reverse the gonadal atrophy but did not interfere with the adrenal hypertrophy. A differential response was also found in rats made severely hypothyroid with propylthiouracil prior to hypothalamic destruction. Ovarian atrophy without concomitant change in adrenal cortex occurred following placing of lesions and goitrogen withdrawal. Evidences of adreno-cortical activation were seen in rats with diabetes insipidus (lesion-induced) and in chronically starved animals with marked hypothalamic deficit. The results signify that basal adenohypophysial ACTH secretion can be enhanced by chronic stress despite arcuate-median eminence lesions and that some aspects of thyro-ovarian interaction may be mediated through a hypothalamic-hypophysial component.

A FUNDAMENTAL role of the hypothalamus in the regulation of pituitary trophic hormone function has been securely established although pressing questions remain unanswered (1,2). The physiological anatomy underlying hypothalamic-hypophysial mechanisms is still obscure and the degree of anterior pituitary hormone secretion which escapes a central mechanism has not been precisely assessed. There is much evidence which suggests that in the rodent the dependency of pituitary-thyroid, adrenal, and gonadal systems on the hypothalamus differs markedly. A succession of recent experiments on the rat has brought into clearer focus the role of the hypothalamus in pituitary-thyroid interplay. It has been shown that anterior hypothalamic lesions selectively interfere with adenohypophysial TSH secretion under either augmented (3–9) or normal demand conditions (8, 10), and that as a consequence thyroid function is reduced. The residuum

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of TSH function in the adenohypophysis after hypothalamic deficit is nevertheless appreciable and the pituitary-thyroid servomechanism remains operative (11, 12). The disturbances which ensue in the genital system after hypothalamic damage are marked; loss of reproductive rhythm with or without gonadal atrophy is frequently observed (13–23). Differential control of the various pituitary gonadotrophins by the hypothalamus is still a matter of conjecture however. In contrast to the gonad, the morphologic consequences to the adrenal from destruction of the hypothalamus are relatively mild (24) although functional impairment of the adrenal cortex is known to exist (25–28). The relative importance of the hypothalamus in ACTH regulation under circumstances of acute versus chronic stress has not been accurately defined. Another unresolved point concerns the locus of adreno-cortical steroid inhibition in the feedback mechanism.

In view of the established importance of the thyroid gland in facilitating certain phases of reproductive activity (29, 30) and influencing adrenocortical physiology (31), the possibility cannot be discounted that some of the histo-physiologic changes in ovary and adrenal of animals with hypothalamic lesions are non-specific consequences of lowered thyroid hormone levels. In a previous study (11) the effect of exogenous thyroid hormone on the hypothalamic-hypophysial-thyroid system of lesioned rats was analyzed in detail, with incidental observations made also on the other endocrine glands. The present investigation is an extension of this basic study and will be concerned primarily with the response of the adrenal and ovary to experimental manipulation of endogenous and exogenous thyroid hormone levels in the operated rat.

MATERIALS AND METHODS

The bilaterally symmetrical lesions were produced in the hypothalami of adult female albino rats by conventional stereotaxic methods (11, 12). The tip of the coagulating electrode was placed 6 mm. anterior to the external auditory meatus and .5 mm. on either side of the sagittal suture, with electrode placement varied in the vertical dimension (.5–1.5 mm. from skull base). A direct current of 2 milliamperes for 20 seconds duration was used. All rats received tap water and a ration of Purina dog checkers *ad libitum*. Operated rats were allowed to recover for at least 10 days before utilized in further experiments. Lesion localization was determined microscopically on Weil and toluidine blue stained coronal sections (12 micra) through the diencephalic area of formalin fixed brains. At various time intervals postoperatively, water intake and urine output were measured over a 72-hour period in rats maintained individually in special metabolic cages. The effects of varying the thyroid hormone level on the endocrine system of operated rats was tested in two major experiments. In the first, rats were treated postoperatively with various dose levels of sodium-*l* triiodothyronine (.4, 2 and 20 μg.) and sodium-*l* thyroxine (10 μg.) made up in 0.9% NaCl (pH. 8.5). The hormones were administered in constant volume (.5 ml.) by daily subcutaneous injections throughout the last two weeks of the post-lesioning period (24–70 days). In the second experiment, rats were made severely hypothyroid prior to electrocautery by feeding

propylthiouracil in the ration (.1%) for 30 days. Goitrous rats were lesioned and immediately returned to the normal diet until sacrifice (11–21 days). The goitrogen was similarly withdrawn from unoperated animals in parallel experiments. At autopsy, the hypophysis, thryoid, adrenal and portions of the reproductive tract were quickly dissected, weighed on a micro-torsion balance, and then placed in suitable fixatives in preparation for histological study. The finer structure of the adenohypophysis was examined on Helly fixed, paraffin embedded serial sections cut at thicknesses of 3 micra through the horizontal plane. Masson staining was used routinely with histochemical stains applied to alternate tissue sections for identification of glyco-protein granulation in the basophils (11, 12). The other endocrine tissues were studied after fixation in Bouin's picro-formol solution and staining with Harris' hematoxylin and eosin or with the Masson stain.

RESULTS

Localization of the lesion

Although there were differences in the site and extent of necrosis, the center of the lesion was usually at the juncture of the anterior and middle thirds of the ventral hypothalamus. Typically, both areas of electrolysis were confluent in the mid-line (Fig. 1). The inclusive area of coagulation extended rostrocaudally from the region of the paraventricular nucleus to the mamillary body (Fig. 2). The degree of damage at rostral and caudal extremes was relatively slight and difficult to assess. In most instances, suprachiasmatic and paraventricular nuclei were spared but ventricular

All figures (except 2 and 10) are photo-micrographs taken with "B" and "G" filters

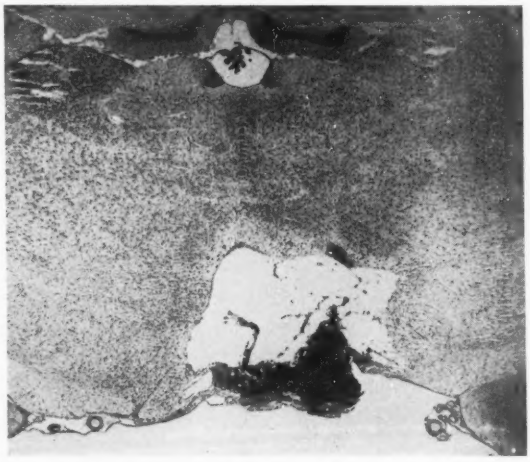

FIG. 1. A coronal section through the diencephalon of an obese (476 gm.) lesioned (56 days) rat which received thyroxine. The floor of the hypothalamus is well vascularized and intact but arcuate nucleus and median eminence are considerably damaged. Thyroid hormone failed to correct the severe ovarian atrophy (paired ovaries, 44 mg.) but induced adrenal hypertrophy (toluidine blue, ×15).

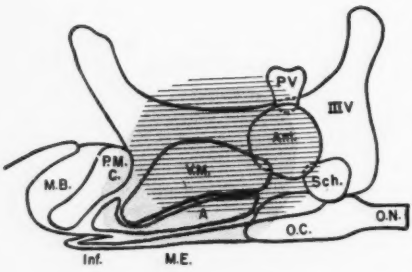

FIG. 2. Diagrammatic representation of the rat's hypothalamus (modified after Krieg, 46; Greer, 2) depicting the major nuclei damaged by electrocautery. Dorsally placed lesions (horizontal hatched area) affected thyroid function without apparent effect on the ovary; both thyroid and ovary were affected with lesions in the ventral hypothalamus (stippled area) IIIV. = third ventricle; O.N. = optic nerve; O.C. = optic chiasm; Sch. = suprachiasmatic nucleus; Ant. = anterior hypothalamic nucleus; P.V. = paraventricular nucleus; V.M. = ventromedial nucleus; A. = arcuate nucleus; M.E. = median eminence; P.M.C. = premamillary nuclear complex; M.B. = mamillary body; Inf. = infundibulum.

(3rd) distension in this region was commonplace (Fig. 3). Damage to the posterior hypothalamus was slight. Some destruction of neighboring fiber tracts (medial forebrain bundle, fornix, mamillo-thalamic) inevitably occurred and relationships of the 3rd ventricle were always disturbed. Lesions deeply placed in the ventral hypothalamus destroyed the periventricular grey area and median eminence, as well as damaging to some extent anterior hypothalamic and dorso- and ventro-medial nuclei. Such lesions produced atrophy of the reproductive tract and disturbances in the pituitary-thyroid system. Lesions more dorsally placed which destroyed dorso- and ventro-medial and anterior hypothalamic nuclei but spared the arcuate nucleus and median eminence affected the pituitary-thyroid system without apparent effect on the ovary. The degree of impairment in thyroid function with either type of lesion was not greatly different (12).

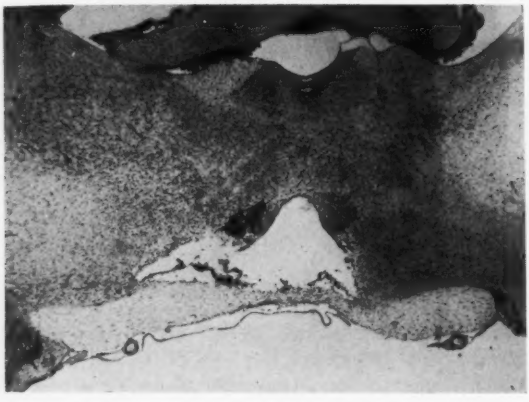

FIG. 3. The degree of damage in the anterior hypothalamus of the rat described in Figure 1. There is ventricular distension and some condensation of cells in the paraventricular nuclei; damage is otherwise relatively slight (toluidine blue, ×15).

TABLE 1. EFFECT OF VARYING THYROID HORMONE LEVEL ON ADRENAL AND OVARIAN ACTIVITY IN RATS BEARING HYPOTHALAMIC LESIONS

Group	No. of rats	Mean body weight (gm.)			Days lesioned	Mean gland weight (mg.)			
		At operation	At initiation of hormone treatment	At sacrifice		Adrenal	Level of significance[2]	Ovary	Level of significance[2]
Expt. 1									
Normal Control Weight	12			386±32[1]	0	59.4±3.5	Pn.s.	103.4±9.3	P<.001
Lesion, Untreated (Obese)	6	273± 7		453±29	60–67	70.4±7.2		57.8±6.4	
Lesion, Untreated (Non-obese)	6	271±13		318±44	60–67	55.3±5.9	Pn.s.	69.6±7.4	Pn.s.
Normal "Age" Control	18			285±40	0	60.6±3.2		74.8±3.4	
Lesion + 10 μg.T_4 (Obese)	8	237± 6	355±32	390±43	48–64	63.7±1.7	P<.005	53.9±5.6	
Lesion + 10 μg.T_4 (Non-obese)	13	246±14	268±34	276±47	48–64	53.2±2.3	P<.001	54.5±5.9	P<.001
Intact+10 μg.T_4	15		308±25	305±23	0	69.8±3.1		93.4±4.9	
Expt. 2									
On PTU, 30 days	11			271±35	0	44.0±3.4		66.8±4.7	P<.05
Lesion, off PTU	18	269±30		240±27	11–21	47.7±2.3	Pn.s.	53.1±5.4	P<.01
Intact, off PTU	18	268±46		275±47	0	48.2±1.9		72.6±5.5	

[1] Mean body weight ± average deviation; in other columns (gland weight) ± values refer to the standard error of the mean.
[2] Statistical significance calculated from "t" test tables. "P" values >.05 are not considered significant (n.s.).

Morphologic Effects of Lesion

The morphologic response of the ovary is statistically evaluated in Tables 1 and 2. Ovarian atrophy was found in both obese and non-obese operated rats but was more severe and occurred more often in the former. The genital hypoplasia was grossly visible in rats with hypothalamic obesity; ovaries were yellow and fibrous in appearance and uteri pale and thread-like. A suggestion as to the efficacy of the lesion was evident from gross inspection of the floor of the hypothalamus at autopsy. With severe atrophy of the reproductive tract, the ventral hypothalamus rostral to separation of the hypophysial stalk from median eminence presented an irregular rather than smooth surface and displayed brown discoloration. The marked regression of the gonad in obese rats became more evident when comparison was made with older intact rats of approximately the same weight ("weight controls") rather than with animals of similar age

TABLE 2. DIFFERENTIAL RESPONSE OF ADRENAL AND OVARY TO TRIIODOTHYRONINE IN RATS BEARING LESIONS

Treatment	No. of animals	Mean body weight (gm.)		Mean organ weight (mg.)			
		Initiation of hormone treatment	At sacrifice	Adrenal	100 gm. final body weight		% change
					% change	Ovary	
Intact—Saline	16	240±31	257±30[1]	22.5± .7		25.9±1.1	
Intact—.4 μg.T_3	14	290±33	287±22	22.9± .7	0	27.0±1.3	+ 4.2
Intact—2μg.T_3	18	234±21	249±18	26.7± .9	+18.7*	36.5±1.5	+40.9*
Intact—20 μg.T_3	15	242±31	238±22	33.0±1.2	+46.6*	38.8±1.7	+49.9*
Lesion—Saline	14	215±29	238±36	21.0±1.1		20.4±1.4	
Lesion—.4 μg.T_3	10	296±24	334±38[2]	17.2± .9	−18.1*	19.8±1.4	− 2.9
Lesion—2 μg.T_3	13	270±34	273±27	27.6±1.4	+31.4*	23.4±1.7	+14.7
Lesion—20 μg.T_3	11	288±34	249±36	36.7±2.6	+74.6*	23.5±2.8	+15.2

[1] Mean final body weight ± average deviation; in other columns ± values represent standard errors of the mear.
[2] Some animals in this group were obese.
* Mean percent changes (from appropriate saline control value) which are highly significant statistically ("P" <.005). Other values are not deemed significant ("P" >.05).

("age controls"). Calculation of mean ovarian-body weight ratios in markedly obese animals yielded values less than 50% of normal; in some, ovarian atrophy approached the degree found after hypophysectomy. Destruction of the arcuate nucleus appeared to be a necessary prerequisite for reduction in weight of the ovary. Damage to the median eminence was an invariable accompaniment but some gonadal atrophy was found with either rostral or caudal portions of this neural structure apparently undamaged.[2]

The histologic changes in the gonad were clearly separable into two major categories. With marked atrophy, the structure of the ovary was preponderantly follicular. Small, medium and large follicles measuring between 500–700 micra in diameter, were present in healthy condition (Fig. 4). There was much atresia, and interstitial gland material, deeply basophilic in staining reaction, was abundant. Wheel-like nuclei were commonplace and deposits of yellow pigment were substantial. Corpus luteum formation was inhibited. When present, corpora lutea were small (less than 500 micra in diameter) and undergoing luteolysis. The second category of histologic alteration from normal was usually found in ovaries of non-obese lesioned rats in which only moderate atrophy occurred. These were characterized by the presence of well developed, histologically active corpora lutea, some over 1 mm. in diameter. In contrast, the follicular apparatus appeared suppressed. Few follicles had developed beyond the early antrum stage and none greater than 200 micra in diameter was visible. Regardless of the histologic picture in the ovary, the uterus was small, avascular and bore a slit-like lumen. The endometrium was either in the diestrous condition or similar to that in the spayed rat (Fig. 5). The luminal epithelium was smooth and of the cuboidal or low columnar type. No mitoses were visible. The uterine glands were collapsed and stained deeply, and fibro-muscular tissue was condensed.

Adrenal morphology was not consistently affected by hypothalamic

[2] The degree of vascular disturbance engendered in the adenohypophysis after experimental manipulation either at hypothalamic or pituitary stalk levels bears importantly on the question of neural regulation of pituitary trophic hormone function (1, 45). In the present experiments, encroachment of some lesions on the primary capillary network in the median eminence was inevitable. The extent of damage at this site was not quantitated by special methods. Several sets of facts subvert the probability that variable damage to this component of the hypophysial portal system was primarily responsible for the major endocrinologic changes which ensued after operation. First, appreciable damage to the primary plexus might be expected to alter substantially the vascular anatomy and histologic integrity of the anterior pituitary; instead, the pars distalis appeared healthy and displayed minimal vascular change with little or no infarction. Secondly, the degree of impairment of the pituitary-thyroid system found after median eminence lesions was little greater than that which ensued when this area was spared. Finally, the post-operative period was of sufficiently long duration to expect that portions of the hypophysial portal system temporarily damaged would have regenerated.

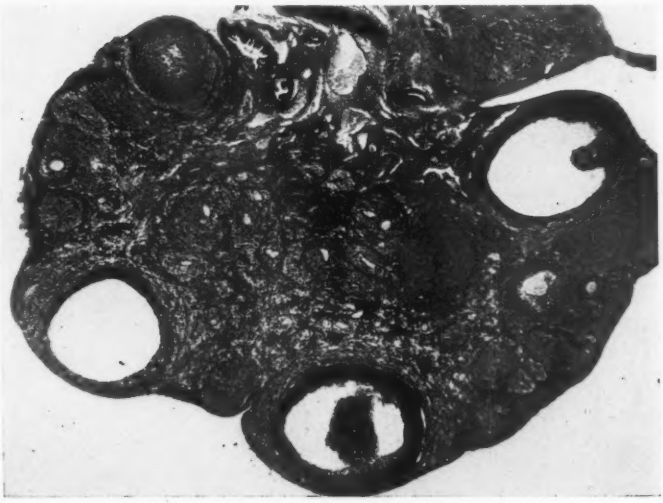

FIG. 4. Follicular character of the atrophic ovary in the untreated rat with lesion (70 days); the interstitium is well developed but no corpora lutea are present (hematoxylin and eosin, ×35).

destruction (Tables 1 and 2). Absolute weight of the adrenal in obese rats could be greater than that in either age or weight controls, with adrenal-body weight ratios not significantly altered. The histologic appearance of adrenal cortex and medulla in the operated rat was variable but no more so

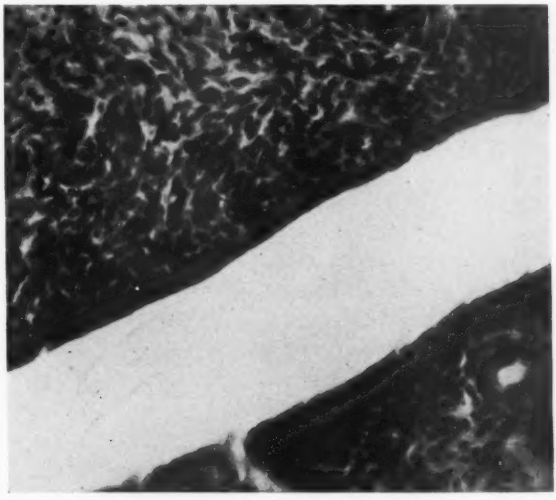

FIG. 5. Typical histologic appearance of the endometrium in the rat described in Figure 4. The regression in epithelium and stroma approximates that found in the spayed animal (hematoxylin and eosin, ×400).

than in intact controls. Several isolated instances of adrenal atrophy did occur but these were associated with adenohypophysial rather than hypothalamic damage.

Effects of varying thyroid hormone level

Thyroid hormone administration produced marked stimulation of the reproductive tract in the unoperated rat. The ovaries were very vascular and enlarged and the uteri grossly hyperemic and swollen (Tables 1 and 2). The ovaries were heavily luteinized, with fresh corpora lutea and those of older orders visible (Fig. 6). Some of the larger corpora lutea were over 1.2

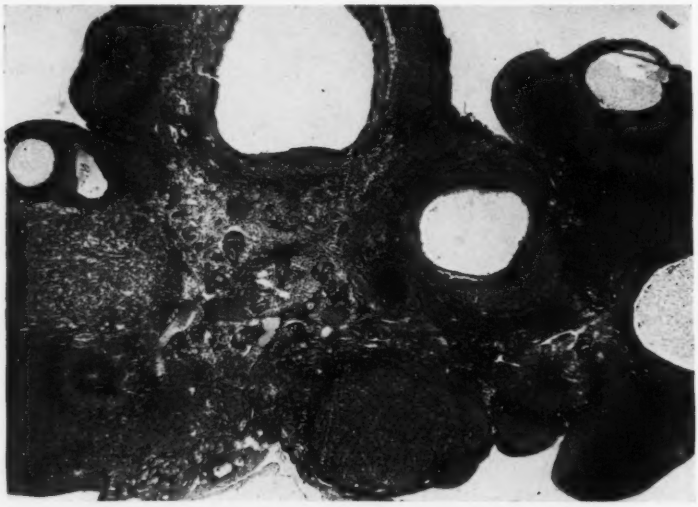

FIG. 6. The morphologic response of the ovary to thyroid hormone administration in the intact rat. The enlarged ovary contains many large follicles and well developed corpora lutea (hematoxylin and eosin, ×35).

mm. in diameter and appeared histologically active. Healthy follicles in all stages of development were present. The uterine and vaginal histological structure closely resembled that seen with continued estrogen stimulation. The tall luminal epithelium in the endometrium contained many mitoses (Fig. 7). The enlarged uterine glands displayed proliferative changes and penetrated deeply into the stroma. There was extensive leucocytic infiltration of stroma and muscles layers. The latter were thickened and extended into the mesometrium. The structural changes in the vagina were indicative of sustained activation. The stratified epithelium was thickened and cornified with numerous mitoses present in the basal layers (Fig. 8). There was appreciable desquamation of the superficial cornified elements.

The histology of the reproductive tract in lesioned rats receiving thy-

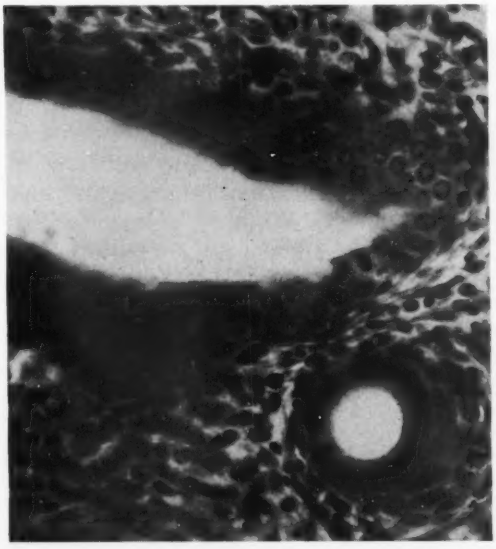

FIG. 7. Uterine hyperplasia which accompanied ovarian stimulation with exogenous thyroid hormone; numerous mitoses are visible in the hypertrophic epithelium (hematoxylin and eosin, ×400).

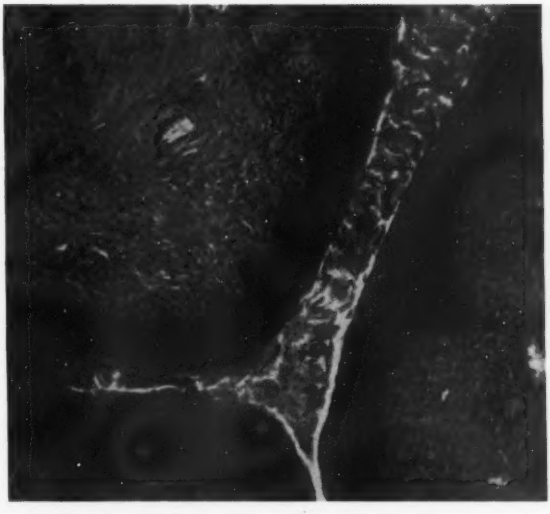

FIG. 8. The vaginal structure corresponding to the gonadal and uterine changes shown in Figure 6 and 7. The thickened epithelium is cornified, with much desquamation of superficial elements into the lumen (hematoxylin and eosin, ×100).

roid hormone contrasted highly with that in the intact animal. The ovarian atrophy was not corrected (Table 1 and 2) and the histology of the gonad appeared essentially unchanged (Fig. 9). The small uteri contained slit-like lumina lined by low columnar or cuboidal epithelium. No glandular activity was evident and the fibro-muscular tissue was condensed. The vaginal epithelium was thin with superficial layers consisting of mucified columnar cells. Luminal concentration of leucocytes was marked.

In contrast to the differential effects on the gonads, exogenous thyroid hormone induced substantial adrenal hypertrophy in lesioned rats which paralleled that found in intact controls. Triiodothyronine at higher dose

FIG. 9. Lack of effect of thyroid hormone on the gonad of a rat with hypothalamic lesion. The histologic picture of the atrophic ovary remains predominantly follicular (hematoxylin and eosin, ×35).

levels was a potent stimulus to adrenal enlargement while inhibiting body weight gain. The 10 μg. dose of thyroxine used was less effective in causing adrenal hypertrophy. It retarded but did not abolish the weight gain in obese animals. The lowest dose of triiodothyronine used (.4 μg.) exerted no significant influence on adrenal weight, nor did it interfere with the development of hypothalamic obesity. The histology of the enlarged adrenal revealed activational changes restricted largely to the fasciculate zone of the cortex with the medulla relatively little affected.

The morphologic response of adrenal and ovary in rats made severely hypothyroid with propylthiouracil prior to hypothalamic lesioning also differed significantly when the goitrogen was withdrawn (Table 1, Experiment 2). Pretreatment with the goitrogen for one month resulted in the anticipated thyroid hyperplasia (3–4 fold) and adrenal atrophy. There was no significant change in weight or histology of the ovary during this regi-

men. In both operated and intact rats adrenal gland weight was still subnormal 2–3 weeks after propylthiouracil withdrawal but evidences of ovarian atrophy were present only in the lesioned group.

A cyto-physiologic and histochemical analysis of the adenohypophyses of most lesioned rats used in this study has been considered in detail elsewhere (11, 12). The additional information derived from this investigation closely conforms. In brief, pituitary infarction was infrequent and aside from sinusoidal distension the vascular architecture was little affected. There was condensation of all cellular types in the parenchyma with many acidophils in advanced stages of degranulation. Significant shifts in the numbers and cytologic activity of beta and delta basophils occurred which correlated well with lesion placement and gonad and thyroid function. The delta basophils responded more sensitively to hypothalamic destruction, displaying marked change in rats with ovarian atrophy. Characteristic clustering of these gonadotrophes along the portal vessels was lost and their glyco-protein granulation was distinctly reduced. Despite the most extensive lesions, the beta basophils (thyrotropes) persisted and these retained their elective affinity for aldehyde fuchsin. Thyroxine and triiodothyronine induced drastic changes in the chromophilic cell types of the adenohypophyses in operated rats. Regranulation and staining intensity of acidophils were increased. Beta cells almost completely disappeared. There was a return of delta basophils to their customary locations in dorsal and ventral regions of the gland. The alterations in the basophilic cell types in thyroid treated lesioned rats appeared qualitatively similar to those in intact animals. (The quantitative implications will be described in another study.)

Effects of diabetes insipidus and starvation

In table 3 are indicated the adrenal gland weights of individual lesioned rats in which water intake and urine output were measured just prior to thyroxine treatment. Group A is comprised of animals in which diabetes insipidus ensued as a consequence of hypothalamic destruction (rats with urine volumes at least twice greater than the mean of unoperated rats were arbitrarily considered to be polyuric).[3] Inspection of adrenal weight values in polyuric (Group A) and non-polyuric lesioned rats (Group B) receiving identical thyroxine treatment reveals that in the former the adrenal is not only well maintained but also has the capacity to enlarge despite damage to the neuro-hypophysis. Adrenal hypertrophy in the lesioned rat was not

[3] In view of the fact that in hypothalamic obesity the metabolic mass is not the same as that of intact rats at similar body weight, some reservation must be held regarding expression of urine output in terms of "cc./hours/body weight." There is no doubt however that a true diabetes insipidus existed and actually may have been more marked than the values indicate.

TABLE 3. ADRENAL WEIGHT RESPONSE TO EXOGENOUS THYROID HORMONE IN LESION-INDUCED POLYURIC RATS

Group	Final body weight (gm.)	Days lesioned	Urinary output cc..100 gm. body weight/24 hr.	Adrenal weight (mg.)
A. Lesioned, Polyuric	332	50	57.0	49.4
	360	51	11.4	71.0
	454	51	10.6	67.5
	404	57	12.5	60.0
	384	58	14.9	56.5
	425	120	8.5	81.4
	400	120	14.5	93.0
	284	120	34.5	65.0
Mean	381		20.5	68.0
B. Lesioned, No Polyuria	250	50	5.5	50.4
	234	51	3.0	57.2
	302	57	5.0	52.0
	266	58	2.7	61.0
	218	210	1.9	44.5
Mean	254		3.6	53.0
C. Mean of 18 Intact "Age" Controls	290	0	3.9	60.6
D. Mean of 11 Intact "Weight" Controls	391	0	3.3	64.8

Urine output was measured just prior to initiation of thyroxine treatment (10 μg. daily, for last two weeks of experiment).

restricted to experimentally induced hyperthyroidism. It occurred in animals which ate little or no food after operation or in those from which food and water were withheld by experimental design. In a group of 14 starved rats sacrificed 6–25 days after electrocautery (28.5% mean body weight loss) all but one responded with either relative or absolute increase in weight of the adrenal (Fig. 10). In 7 of these rats, adrenal weight was greater than it would be prior to starvation.

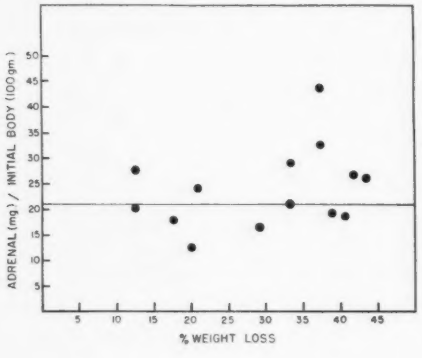

FIG. 10. Effect of starvation on adrenal hypertrophy in lesioned rats. The horizontal line represents the mean adrenal-body weight ratio in normally fed rats (225–275 gm. body weight). Values for individual operated rats (circles) subjected to chronic starvation are related to the pre-starvation rather than final body weight. The adrenal hypertrophy is plotted as a function of body weight loss (%) regardless of the number of days the rat was lesioned.

DISCUSSION

The association of gonadal atrophy and hypothalamic obesity has been noted often; the relationship is not causal and different mechanisms are involved (32, 33). In this study, the genital hypoplasia was clearly referable to secretory alterations in the adenohypophysis which were not contingent on the presence or absence of obesity. Similar interpretation was made by Bogdanove and associates (6, 7) and Van Dyke *et al.* (23) in the male and female rat respectively. The severity of the ovarian atrophy which followed destruction of the periventricular grey area and the median eminence pointed to these neural structures as being of crucial importance in the hypothalamic-hypophysial pathway governing structural and functional integrity of the ovary. That varying degrees of gonadal atrophy, with or without disturbances in reproductive rhythm, result from generalized damage to the tuberal area in the rat has been reported (16, 17, 23, 34) but the relationship of this hypothalamic region to a more rostral one controlling the sex cycle is rather obscure. With anterior hypothalamic lesions, disturbances in cyclicity without gonadal atrophy occur. It was found in both guinea pig (13–15) and rat (18–20) that destruction of the area from optic chiasm to rostral edge of the median eminence produced persistent estrus, hypertrophied uteri and markedly follicular ovaries in which corpus luteum formation was inhibited; gonad weight was unchanged.

The hormonal alterations responsible for the imbalance in the pituitary-ovarian system after hypothalamic damage have not been elucidated. Dey (15) and Hillarp (18) postulated that anterior hypothalamic lesions curtailed LH secretion while sparing the FSH mechanism in the adenohypophysis, thus permitting continued estrogen secretion from the follicular ovaries. The induction of corpus luteum formation in such ovaries with luteinizing hormone preparations (23, 35) reinforces the view that pituitary LH secretion is deficient in the lesioned animal but selective failure in LH function cannot explain some of the reproductive phenomena which result. Greer (19) was able to re-establish normal cycles and corpus luteum formation with progesterone administration in persistent estrus rats bearing anterior hypothalamic lesions; pseudopregnancy was also produced upon cervical stimulation. The closest counterpart to this in the intact animal was described by Everett (36) in a spontaneous estrus strain in which correctly timed injections of sub-inhibitory quantities of progesterone restored regular ovulatory cycles. If the possibility of hypothalamic re-organization in Greer's experiments is discounted, it would appear that balanced gonadotrophic hormone secretion by the pituitary can be restored by progesterone in the absence of the hypothalamic stimulus and despite the fact that some effects of this ovarian hormone are known to be mediated through the central nervous system. The crucial element in the hypothalamic-hypophysial-gonadal interplay may involve primarily an estrogen-FSH

mechanism with changes in LH secretion secondary. In a series of trenchant observations, Flerko (20, 21, 22, 47) demonstrated that a region in the hypothalamus just ventral to the paraventricular nucleus is sensitive to changing levels in circulating estrogens and constitutes an essential link in in the reciprocal interaction between ovary and adenohypophysis. In rats with this area destroyed much more estrogen than normal was required to inhibit pituitary FSH secretion. Conversely, ovarian autografts in the same region resulted in reduced uterine weight and diestrus, responses indicative of FSH suppression. Grafts at other sites (posterior hypothalamus, hypophysis and liver) were ineffective. The results were considered to disprove the concept that estrogen inhibits FSH secretion directly at the adenohypophysis. It must be emphasized that although the effects of ovarian hormones on gonadotrophic hormone functions are mediated in part through neural mechanisms, direct action at pituitary levels is not necessarily excluded. Rose and Nelson (37) evoked substantial cytologic change in the pars distalis of rats with local administration of estradiol into the hypophysial fossa, a finding consistent with the classic interpretation of a pituitary-ovarian feedback mechanism.

The best interpretation that can be made from the present results is that destruction of the arcuate nucleus-median eminence complex seriously interfered with both FSH and LH secretion by the anterior pituitary. Although vaginal smears were not made in lesioned rats, the histologic picture of the reproductive tract was consistently one of atrophy. An absolute deficiency of estrogen secretion must have occurred in all atrophic ovaries irrespective of corpus luteum inhibition or apparent sparing of the follicular apparatus. The histochemical changes in the adenohypophysis conformed closely. The decreased size and number of growing follicles and the abundance of wheel-cell formation in the interstitial tissue of severely atrophic ovaries correlated strongly with drastic reductions in both central and peripheral gonadotrophs in the pars distalis. The totality of histophysiologic change in the pituitary-ovarian system resulting from arcuate nucleus-median eminence lesions simulated that which ensues from pituitary transplantation or severance of its stalk. Whether or not luteotrophin continues to be secreted after the placement of lesions which curtail FSH and LH secretion remains to be tested. There is impressive evidence that vigorous and prolonged luteotrophin secretion does not require an intact hypothalamic connection, even though some modulation of hormone secretion by the hypothalamus is quite probable. Luteotrophin is secreted from rat pituitaries transplanted to the kidneys very likely instead of other gonadotrophins, and in amounts sufficient to maintain functional corpora lutea for months (38).

The manner in which the anterior and tuberal hypothalamic "centers" of the rat are integrated to achieve balanced gonadotrophic hormone secre-

tion from the anterior hypophysis defies analysis at present. Without direct measurement of the levels of the individual gonadotrophins in blood and pituitary of lesioned rats, differential regulation of FSH and LH secretion by either hypothalamic zone cannot be assessed. It would be expected that stimuli from the rostral hypothalamus—whatever their nature—should inevitably converge in or near the median eminence region as a focal point in the hypothalamic-hypophysial pathway for gonadotrophic hormone regulation. Destruction of median eminence and associated periventricular grey would therefore eliminate all hypothalamic influence regardless of its source and should lead to the greatest gonadotrophin deficit and gonadal atrophy. It is significant that in the male rat severe gonadal atrophy also occurs after destruction of the arcuate nucleus-median eminence complex (7, 25) and that under these circumstances pituitary gonadotrophin is drastically reduced (7, 39). The fact that gonadotrophic hormone stores in the pituitary can be decreased even in lesioned rats with testes of normal size (7, 39) underscores the difficulty in interpretation of the relative importance of the alleged separate gonadotrophic centers in regulation of gonadotrophic hormone secretion. Classic neuro-anatomic technics may have to be employed to evaluate more precisely the extent of damage in the hypothalamus, even in apparently unaffected areas.

The failure to induce impressive adrenal atrophy in the female rat after electrocautery of the hypothalamus has been general (2, 16, 17, 18, 34). In the male, marked adrenal atrophy can be produced with median eminence lesions (27) but it is usually not selective, being associated frequently with pan-hypopituitarism (6, 7, 39). The absence of morphologic change in the adrenal does not preclude the strong probability that some impairment of adreno-cortical function occurred in untreated lesioned rats of this study. McCann and his associates have demonstrated that median eminence lesions can block acute responses of the adrenal cortex without atrophy (25), will suppress compensatory hypertrophy of the gland to unilateral adrenalectomy (26) and may inhibit the rise in ACTH content of blood and pituitary which attends adrenalectomy (27, 28). The fact, however, that adreno-cortical hypertrophy can be induced with such stressful procedures as starvation or chronic thyroid hormone treatment—both potent activators of the pituitary-adrenal system (40, 41)—strongly suggests that the pituitary can augment its basal ACTH secretion even after extensive hypothalamic lesions. It has also been shown, as in previous work (5, 25), that lesion-induced diabetes insipidus and adrenal hypertrophy are not mutually exclusive. Evidence of adreno-cortical stimulation in rats with severe neuro-hypophysial damage indicates at best a limited role of the neurohypophysial hormones in facilitating SUSTAINED ACTH release by the anterior pituitary, a conclusion equally applicable to TSH function as well (11, 12).

The finding that exogenous thyroid hormone engenders adrenal hypertrophy without reversing the gonadal atrophy reveals that the effects of hypothalamic damage on the target endocrine glands are not merely nonspecific consequences of lowered metabolism. Brooks, Marine and Lambert (42) induced hypermetabolic states in lesioned rats with thyroxine but did not study the histo-physiologic consequences to the endocrine system. As in their study, thyroxine administration was found to retard but not abolish the weight gain in operated animals. The stimulation of the reproductive tract in the intact rat receiving thyroxine or triiodothyronine cannot be satisfactorily explained. Maqsood (29) and Reineke and Soliman (30) have evaluated the many discrepant reports which nevertheless strongly implicate the thyroid gland in mammalian reproductive processes. Experimental procedures, which in themselves drastically altered thyroid hormone levels far beyond physiologic limits, have tended to obscure the possible role of the thyroid in gonadal function. In the present study, this factor was taken into account. With hyperthyroid states ranging from mild to moderate and severe, there was marked stimulation of the reproductive tract in the intact rat. The effects on the uterus and vagina were much like those induced with chronic estrogen administration.

Several physiologic mechanisms exist through which thyro-ovarian interaction may conceivably occur; namely, 1) an indirect effect mediated via the adrenal, 2) a change in excretion rates of gonadotrophic and ovarian hormones or altered sensitivity of target tissues to them, and 3) a direct effect of thyroid hormone on the pituitary-ovarian axis with or without involvement of the hypothalamus. Inasmuch as the adrenal hypertrophy in operated rats was equivalent to that of intact controls, it is not deemed likely that differential response of the ovary was due to differences in adrenocortical hormone levels. Differential excretion or inactivation of gonadotrophic hormones and threshold differences in the gonad are known to occur in hyperthyroid rats (43). That such changes were restricted to the intact and not the lesioned animal despite similar thyroid hormone treatment over a range of dose levels is implausible. More relevant may be the fact that thyroid hormone in amounts inducing relatively mild hyperthyroidism is known to augment certain phases of gonadotrophic hormone secretion by the pituitary (29, 44). The substantial decrease in the numbers and cytologic activity of the delta basophils in the adenohypophyses of untreated lesioned rats and their considerable increase in the pituitary of the unoperated animal receiving thyroid hormone are consistent with this thesis. The persistence of gonadal atrophy in the operated animal could reflect irreparable damage to the mechanisms of adenohypophysial FSH and LH secretion. The restoration of delta cells in the adenohypophysis with exogenous thyroid hormone renders this interpretation questionable, however, and suggests further that thyroid hormone can act at both hypothal-

amic and pituitary levels. The reappearance of these cells at a time when TSH content of the anterior pituitary is far below normal excludes them as the producer of TSH (12) but whether their return signifies production of gonadotrophins without concomitant release, or even augmented ACTH secretion in view of the adrenal enlargement, cannot be resolved with the data at hand. The influence of the thyroid on the hypothalamic- hypophysial-ovarian system must therefore remain speculative at present.

Acknowledgment

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THE EFFECTS OF EPINEPHRINE ON THYROXINE METABOLISM: IODINE EXCRETION AFTER VARIOUS THYRONINE DERIVATIVES[1]

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ABSTRACT

Epinephrine was found to increase urinary I^{131} (primarily as iodide) after the administration of labeled L-thyroxine, D-thyroxine and L-triiodothyronine as compared to the I^{131} excretion after injection of these thyronines without epinephrine. Epinephrine had no effect on the excretion of isotope after the administration of radioactive iodide. Fecal I^{131} from these sources was slightly lowered by epinephrine. Other thyronine derivatives were studied without epinephrine and were found to have varying rates of excretion.

SWANSON has shown that the increased oxygen consumption produced in rats by epinephrine is thyroid-hormone-dependent (1). This effect is of short duration, oxygen consumption returning to normal within four hours. It appeared possible that this effect is related to an epinephrine effect on the metabolism and/or distribution of thyroid hormone. It has been shown that epinephrine produces a lowering of the serum protein bound iodine in rats and humans (2, 3, 4, 5).

In the present study the effects of epinephrine on iodine excretion were determined. Thyronine derivatives labeled with I^{131} were administered to thyroidectomized rats which had been withdrawn from replacement therapy (3.5 μg. L-thyroxine/100 gm. body weight/day) one month prior to the start of these experiments. This was done to lower the body pool of thyroid hormone to a level at which the injected labeled material would represent essentially all the thyroid hormone available to the organism.

Since metabolic rates were not determined, it is not known if the amounts of thyroactive compounds used in this work (2–4 μg.) supported the stimulatory effect of epinephrine on oxygen consumption. Although Swanson found increased oxygen consumption after epinephrine administration to rats receiving daily injections of 3 μg. of thyroxine, it was not the purpose of the experiments reported here to necessarily cause hypermetabolism. It was assumed that, even in the absence of a demonstrable effect on oxygen consumption, an effect of epinephrine on thyroid hormone metabolism would be exerted on thyroactive material administered as a single dose.

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In addition to the studies utilizing epinephrine, the excretory patterns of radioactive tetraiodothyroacetic (Tetrac) and tetraiodothyropropionic (Tetraprop) acids and of triiodothyroacetic acid (Triac) were determined.

MATERIALS AND METHODS

Male Long-Evans rats which had been thyroidectomized with I^{131} were used.[2] Groups of five rats were given a single intraperitoneal injection of the thyronine derivatives in amounts of 2–4 μg., incorporating 75–85 μc of radioactivity.[3] The animals were then placed in metabolism cages and urine and feces were collected over 24 or 48 hour intervals for varying periods of time.

The urine was pooled, filtered and made up to a standard volume. The feces were also pooled, homogenized and made up to volumes. Radioactivity in aliquots of both urine and feces was determined in a scintillation well counter. Total counts in the pooled urine and feces were compared to those in a standard containing the amount of radioactive material given to each rat.

In the studies utilizing epinephrine,[4] it was administered intramuscularly at a dose of 80 μg. per 100 gm. body weight. This single injection was made immediately after the administration of labeled thyroid hormone. Compounds which were studied both with and without epinephrine were given to the same group of five animals in both experiments.

Four rats were given I^{131} -iodide (60 μc). After 48 hours two of these animals were given epinephrine. Urine and feces of the four rats were collected during the initial 48 hours; they were collected as two pools for the subsequent 24 hours.

In some of the studies additional aliquots of urine and feces were acidified and extracted three times with equal volumes of n-butanol. The pooled butanol was then washed twice with Blau's reagent (4N NaOH containing 5% Na_2CO_3), which extracts iodide and iodinated tyrosines but not iodinated thyronines. Radioactivity in the butanol was determined before and after Blau's extraction.

RESULTS

The amounts of urinary and fecal radioactivity are tabulated in Table 1. Increased urinary I^{131} was found after epinephrine administration with L-thyroxine, D-thyroxine and L-triiodothyronine but not with iodide. Epinephrine produced a slight decrease in fecal I^{131} with the three thyronine derivatives.

Urinary I^{131} was primarily in the form of iodide; fecal I^{131} behaved mainly as thyronine derivative in its solubility characteristics. Fecal excretion was very low (<4%) in all the rats given isotopic iodide. Table 2 contains the results of butanol and Blau's reagent extractions; Blau's insoluble material has been recorded as "thyronine-like."

The urinary excretion of isotope was greater with triiodothyronine and D-thyroxine than with L-thyroxine. This was true also of Tetraprop and

[2] Two treatments of 500 μc were used; histological examination of similarly treated rats showed no thyroid tissue in the throats or chests.

[3] Radioactive materials were purchased from Abbott Laboratories, Oak Ridge, Tennessee.

[4] "Adrenaline-in-oil," 1:500, Parke, Davis and Co.

TABLE 1. URINARY AND FECAL I^{131} [1]

Material injected	Urinary I^{131}								Fecal I^{131}							
	Time (hours)								Time (hours)							
	24	48	72	96	120	132	168	Total	24	48	72	96	120	132	168	Total
L-thyroxine	14	8		5				27		43		12				55
L-thyroxine & epinephrine	20	11	10	8				49	21	13	9	4				47
D-thyroxine	29	10	3					42	34	12	2					48
D-thyroxine & epinephrine	34	19	5					58	28	7	3					38
Triiodothyronine	25		15					40	46		15					61
Triiodothyronine & epinephrine	47	25						72	29	9						38
Triac	25	5	2	1				33	41	10	1	1				53
Tetrac	9	4		4	2		3	22	6	7		10	5		8	36
Tetraprop	18	8	4			11		41	11	17	12			18		58
Iodide	61	11	5					77	2	1	<1					<4
Iodide & epinephrine[2]			4					76			<1					<4

[1] Data recorded as per cent of administered radioactivity. Each figure represents the average excretion per rat as calculated from the pooled excreta of five rats.
[2] Epinephrine administered at 48 hours.

triac. Only tetrac was deiodinated more slowly than L-thyroxine. The increase in urinary radioactivity over the entire collection period following epinephrine was, with D-thyroxine—38%, with L-triiodothyronine—80% and with L-thyroxine—82%. Radioactivity derived from triiodothyronine was completely excreted within 48 hours when epinephrine was given. A longer collection period was required for essentially complete excretion with epinephrine and the two thyroxine isomers.

TABLE 2. PERCENTAGE OF THYRONINE-LIKE I^{131} IN BUTANOL EXTRACTS

Material injected	Urine	Feces
L-thyroxine	2	90
L-thyroxine & epinephrine	3	83
D-thyroxine	1	83
D-thyroxine & epinephrine	2	79
Tetraprop	2	86
Triac	1	58
Iodide	2	—

DISCUSSION

From the data presented above, it appears that the rates of deiodination of the various thyronine derivatives are not related to their *in vivo* activities. Thus, Tetraprop, triac and D-thyroxine, which are relatively inactive in mammals, were deiodinated more rapidly than L-thyroxine. The more active compound, L-triiodothyronine, was also deiodinated more rapidly than L-thyroxine. Tetrac, a less active material, was deiodinated more slowly than L-thyroxine.

Epinephrine effected a marked increase in urinary I^{131} with the three thyronine derivatives tested. It did not increase the excretion of isotope when given 48 hours after the administration of radioactive iodide, indicating that the epinephrine effect is not exerted on the release of iodide from the tissues. Furthermore, the increased urinary I^{131} after thyronines and epinephrine persisted for 48–72 hours. It is questionable if an epinephrine effect on renal action would persist for this length of time.

The decrease in fecal iodine after epinephrine may result from the lowered concentration of circulating labeled material. Specific differences in the rate of biliary excretion may exist, however. Thus, Tetrac was excreted at a low rate in both urine and feces. Triac, on the other hand, was rapidly excreted via both routes.

Further work on this problem will be designed to determine the mechanism by which epinephrine increases the urinary excretion of iodide derived from thyronine derivatives.

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CHARACTERIZATION OF ESTROGENS IN THE BOVINE[1,2]

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ABSTRACT

Estrone, estradiol-17β and estradiol-17α were identified in bovine placental extracts by chromatography on paper, formation of acetates, Kober reaction, bioassay and absorption spectra in sulfuric acid. A $FeCl_3$-$Fe(CN)_6$ reacting compound with a migration rate on paper chromatograms similar to estradiol-17β was detected in extracts of ovaries from non-pregnant cows but in quantities too low for further identification.

Maternal adrenals and ovaries and fetal adrenals and testes from two cows pregnant 256 and 265 days did not yield detectable quantities of estrogens. The placenta appears to be the principal source of estrogens in the pregnant bovine.

INTRODUCTION

INFORMATION on the natural estrogens of the bovine is limited to identification of estrone in adrenal tissue (1), estrone and estradiol-17α in urine from pregnant cows (2, 3) and estrone in the bile of pregnant cows (4). Nonspecific assays of urine by biological (5) and fluorometric (6) methods have indicated that estrogen levels are markedly increased in the pregnant cow as compared with the non-pregnant cow.

Estradiol-17β and estrone have been demonstrated in the sow ovary (7, 8). The principal source of estrogens in pregnant women is considered to be the placenta from which estrone, estradiol-17β, and estriol have been isolated (9, 10, 11).

The purpose of this study was to determine the identity and principal sources of the estrogens in the bovine.

MATERIALS AND METHODS

Tissue Sources

Ovaries from nonpregnant cows and heifers with grossly normal reproductive tracts were obtained within 15 minutes after slaughter. The ovaries were pooled in groups of 8, 11, and 35 ovaries and assayed by the procedures described below.

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Two Jerseys were sacrificed during late pregnancy. No. 2223 was pregnant 256 days with her first calf and No. 2192 was pregnant 265 days with her third calf. Placentae, ovaries, and adrenals of the cows and the adrenals and testes of their fetal calves were removed for assay.

Placentae were obtained at slaughter from cows in different stages of gestation and from cows following parturition.

Chemical Procedures

All tissues were homogenized and extracted with ethyl acetate in a Waring blendor. The lipid extract was evaporated to dryness in a water bath at 45° C under reduced pressure. Fats were removed by taking up the lipid residue in 70% methanol and then cooling overnight at minus 5 to 10° C. The precipitated fat was removed by centrifugation or filtration in the cold. In the case of samples from cow No. 2192 and a few placental extracts, fat was removed by partitioning between 70% methanol and Skellysolve B. The aqueous fraction, after evaporation of the methanol in a water bath at 45° C under a stream of N_2, was extracted with ethyl acetate. The ethyl acetate was dried over Na_2SO_4 and evaporated to dryness in a water bath at 45° C under a stream of N_2. The resulting residue was chromatographed on paper in the formamide/Skellysolve B system (12) for 4 to 5 hours. The chromatograms were dried in air and the areas within 4 cm. of the origin eluted with methanol. After evaporation of the methanol, the residue was chromatographed in formamide/Skellysolve B-benzene for 5 to 6 hours. After drying the chromatogram, the estrogens were detected with $FeCl_3$-$K_3Fe(CN)_6$ reagent. The origin was eluted as described above and rechromatographed in the benzene/55% methanol system for 24 hours in order to detect estriol if present.

Identification Procedures

When sufficient quantities of a compound were available, the compounds were rechromatographed for various periods of time in formamide/Skellysolve B-benzene. Monoacetates were formed by reacting with one part acetic anhydride to 50 parts pyridine (13). The acetylated estrogens were chromatographed in formamide/Skellysolve B till the solvent front had moved approximately 40 cm. The acetylated estrogens were detected on the chromatograms by the procedure suggested by West (14). Kober tests were run with Hunt's (15) microadaption of the Brown (16) procedure. Four point bioassays, using the 6-hour uterine weight method of Astwood (17) were calculated with covariance as suggested by Bliss (18). Absorption spectra were determined after reacting the compounds with concentrated sulfuric acid for 2 hours. Although this study was not a quantitative one, crude estimates were made by visual comparisons of the dyed spots on chromatograms from tissue extracts and standards.

RESULTS

Up to 35 ovaries in a group were extracted but no estrogen could be completely identified. In all cases, a faint spot was detected on the chromatograms corresponding to estradiol-17β. The quantities were less than 10 μg. and insufficient for further identification. No other phenolic compounds were detected in the ovarian extracts.

Three $FeCl_3$-$Fe(CN)_6$ reacting compounds were detected in the placentae from the two Jersey cows sacrificed during late pregnancy. No phenolic compounds were detected in the ovaries and adrenals of these cows, nor was any found in the adrenals and testes of their fetal calves.

$FeCl_3$-$Fe(CN)^6$ reacting compounds from placental extracts were pooled for further identification. Estrone was identified by migration rate on paper chromatograms, by migration rate of the acetate, by Kober chromogen, and by a significant response in the uterine weight bioassay corresponding to authentic estrone.

Estradiol-17β was identified by migration rate on paper chromatograms, by migration rate of the monacetate, by Kober chromogen, and by significant response in the uterine weight bioassay parallel to authentic estradiol-17β at 1/10 the concentration of estrone.

Estradiol-17α was identified by migration rate on paper chromatograms (Fig. 1), by migration rate of the monoacetate, by Kober chromogen, and by sulfuric acid spectra of the monoacetate.

Estriol could not be detected in any of the extracts.

Table 1 shows the approximate quantities of the three estrogens from the placentae of the two pregnant cows. Relative amounts of estradiol-17α have been higher in term placentae. Estradiol-17β was detected in placentae from the other cows as early as the fifth month of pregnancy.

TABLE 1. APPROXIMATE[1] QUANTITIES OF THREE ESTROGENS IN TWO PLACENTAE

Cow	Tissue weight	Estrone	Estradiol-17β	Estradiol-17α
	gm.	μg.	μg.	μg.
2223	1400	20	30	5
2192	1300	15	30	10

[1] Visual comparisons from spots on paper chromatograms.

DISCUSSION

The placenta of the bovine, like that of the human, appears to be the principal source of estrogens during pregnancy. Estrone and estradiol-17β are found in the placentae of both species. The estrogens in the placentae differ, in that estradiol-17α but not estriol was found in the bovine. The reverse is true for the human (11). It is interesting that estradiol-17α is apparently a major urinary metabolite in the bovine (3) and estriol is considered the principal urinary metabolite in the human (19). Levy, cited by

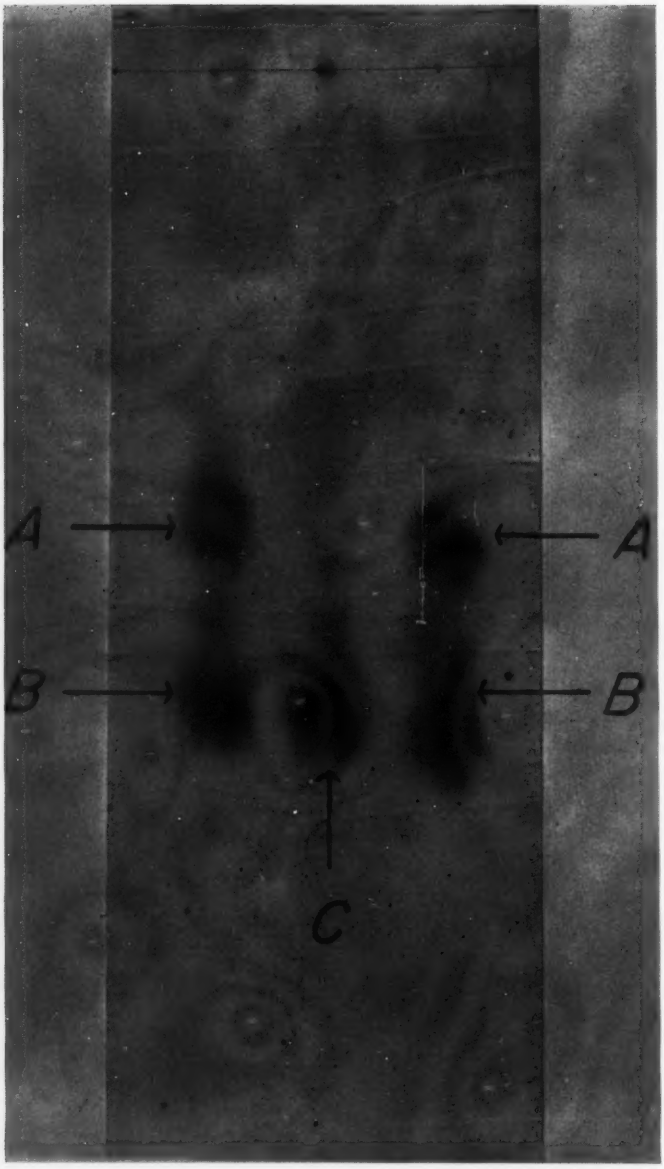

FIG. 1. Paper chromatogram after development with $FeCl_3$-$K_3Fe(CN)$ reagent. (A) Estradiol-17β standard, (B) Estradiol-17α standard, (C) Pooled placental estradiol-17α.

Dorfman (20), has reported that estrone is metabolized to estradiol-17α by bovine blood. This mechanism could account for a part or all of this compound in the highly vascular placental tissue.

A previous paper from this laboratory (21) confirms Short's (22) report that the bovine placentae does not contain progesterone or Δ^4 pregnene-20β-ol, 3-one. Also, testosterone and androstendione were not detected (21). One or more of these steroids, which are possible estrogen precursors, have been reported for the ovary, adrenal, testes, and human placenta. In light of this, bovine placental tissue might be of interest for studies of estrogen biosynthesis.

The low amount of estrogens in the bovine ovary is consistent with reports of low urinary excretion of estrogens in non-pregnant cows (5) and the relatively low amount of exogenous estrogen necessary to bring ovariectomized heifers into estrus (23). Although the data presented here are not conclusive, estradiol-17β is possibly the ovarian estrogen in the bovine.

Failure to detect estrogens in the maternal adrenal, fetal adrenal or fetal testis may be due to lack of sensitive enough methods but does indicate that these organs are minor sources as compared to the placenta.

The relatively high quantities of estrogens in late pregnancy placenta, coupled with rather constant levels of progestins in the ovaries (21) and blood (24) of the bovine, is in contrast to the increasing levels of both groups of steroids in the human placenta during gestation (25, 26).

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FURTHER EVIDENCE FOR MEDIATION BY HISTAMINE OF ESTROGENIC STIMULATION OF THE RAT UTERUS[1,2]

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ABSTRACT

Solutions of histamine dihydrochloride or of a synthetic histamine releasing agent (Compound 48–80) were introduced into the uterine lumen of adult, castrated rats. Within four hours following administration, increases in water content and changes in gross vascular appearance of the organ were produced which were comparable to the maxima induced by intravenous or intraluminal estrogen for the same period of treatment. Intravenously administered cortisol substantially diminished the hyperemia and water imbibition elicited by 48–80 or by histamine.

Two of the 4 structurally interrelated antihistamines tested (Benadryl hydrochloride and Chlor-Trimeton maleate), were effective in blocking estradiol-stimulated hydration and hyperemia.

These findings are regarded as support for the view that estrogen action, in effecting vasodilation and edema in the rat uterus, is mediated by the local release of histamine. It is proposed that cortisol may antagonize responses to estrogen in the uterus by non-specifically decreasing capillary permeability in that organ.

IN EXPERIMENTS reported earlier from this laboratory (1), it was observed that a single intravenous dose of estradiol-17β administered to the ovariectomized rat was followed in 4 hours by a decrease in the histamine concentration of the uterus. The loss was determined to be approximately 17% of the basal concentration in the organ and was shown to be neither a consequence of the usual water accumulation nor a reflection of an estrogen-induced acceleration of histaminase activity. In addition, uteri taken from intact animals during periods of high endogenous estrogen secretion exhibited less histamine than those in quiescent states. On the basis of these observations it was proposed that certain early correlates of estrogen stimulation, particularly vasodilation accompanied by increased

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capillary permeability, may be attributed to the action of histamine released *in situ* by estrogen.

Results of experiments designed to test further this hypothesis are included in the present report. It has been demonstrated that instillation of histamine or of a histamine liberator, Compound 48–80, into the uterine lumen of the ovariectomized rat simulates the action of estrogen in eliciting hyperemia and hydration of the organ. When induced by estrogen itself, these responses could be significantly depressed by administration of the antihistamines, Benadryl hydrochloride and Chlor-Trimeton maleate.

Cortisol administration suppressed the vascular and hydration responses elicited in the uterus by histamine and 48–80.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats, 4 to 7 months of age and averaging 300 gm. in weight, were used. All animals were bilaterally ovariectomized 14 to 16 days before treatment. In one experiment the animals were also bilaterally adrenalectomized just prior to injection. Water and Purina laboratory chow were fed *ad libitum*. While the animals were under light ether anesthesia, solutions of diphenhydramine ("Benadryl")-HCl, pyranisamine ("Neo-Antergan") maleate, tripelennamine ("Pyribenzamine")-HCl or chlorprophenpyridamine ("Chlor-Trimeton") maleate, Compound "48–80," histamine dihydrochloride, estradiol-17β, or their control solutions were injected directly into the lumen of one or both uterine horns. Ligatures were placed immediately above the site of injection. Although varying concentrations of the above substances were employed, the volume injected per horn was kept constant at 0.02 ml. This quantity of fluid did not discernibly distend the uteri of most of the animals used.

In the antihistamine experiments, estradiol-17β in isotonic saline solution (2) was administered intravenously in the amount of 0.5 μg. (0.5 ml.) per 100 gm. body weight unless otherwise stated. From 2.5 to 4.0 mg. of cortisol acetate in aqueous suspension (25 mg./ml.) was administered per 100 gm. body weight to some of the animals receiving intraluminal histamine or Compound 48–80. The hormones were given under light ether anesthesia, each in a single dose via the saphenous vein immediately preceding intrauterine instillation of the non-steroid substances mentioned.

At time intervals following injection, each animal was stunned by a sharp blow at the base of the skull and bled by decapitation; uterine horns were removed (1) and analyzed for water content as previously described (2).

RESULTS

Compound 48–80 and uterine hydration

Naturally occurring and synthetic materials possessing the capacity to cause the release of endogenous histamine have been extensively studied by the pharmacologists (3). Among the more powerful of these is a substance designated Compound 48–80 (3, 4), the exact structure of which is unknown but is believed to consist of molecular chains of an indeterminate number of P-methoxy-N-methyl-phenylethylamine units. Injection of this material at various dose levels directly into the lumen of each uterine horn resulted 4 hours later in the increases in uterine water uptake shown in Figure 1. A dose of 0.2 mg./horn can be seen to produce a water uptake

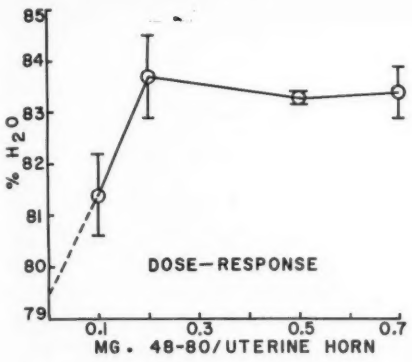

FIG. 1. The effect on uterine hydration of increasing amounts of 48-80 injected directly into the lumen of each uterine horn. Each point represents the mean ± the standard deviation of values obtained from 4–5 animals sacrificed 4 hours after injection.

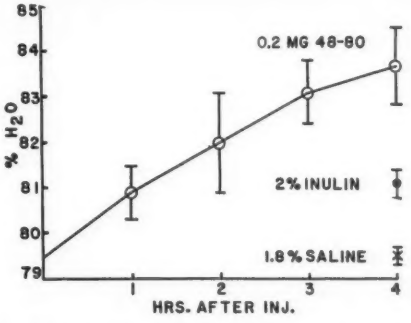

FIG. 2. The influence of time after administration of Compound 48-80 on uterine water content. The histamine releaser was injected intraluminally in the amount of 0.2 mg. per uterine horn. Values for inulin and saline given by the same route are shown for the 4 hour time period. Each point represents the mean ± the standard deviation of values obtained from 4–6 animals.

approaching the maximum attainable with intravenous estrogen (*vide infra*). The local route of administration was employed because 48–80 was observed in preliminary experiments to be toxic when given by systemic routes. Intra-uterine dosages in excess of 0.2 mg./horn were increasingly toxic to the animals generally and also caused extensive vascular congestion in the uterus. Uterine horns exposed to 0.1–0.2 mg., however, displayed a gross appearance indistinguishable from those of intact animals in proestrus, or of ovariectomized rats given estrogen.

A time study utilizing the 0.2 mg. dose of 48–80 is depicted in Figure 2. Inulin was utilized in this experiment as a control for osmotic effects that might result from intraluminal administration of a complex substance such as 48–80. It may be noted that inulin at the 4 hour level caused a water uptake which was, however, significantly below that produced by 48–80 at the same time interval. Thus, a portion of the hydration produced by the histamine releaser may be an osmotic effect of the concentrations employed. Nevertheless, uteri injected with 2% inulin or 1.8% saline showed no hyperemia or gross edema, in contrast to those exposed to 48–80.

An effort was made to block with cortisol the uterine hydration induced by Compound 48–80. Concomitant administration of intra-uterine 48–80 (0.2 mg./horn) and of 3.5 mg. of cortisol acetate per 100 gm. of body weight via the saphenous vein resulted 4 hours later in a significant suppression ($P < 0.01$) of the water uptake observed in the presence of the histamine liberator alone (Fig. 3). For purposes of comparison, data depicting the

hydration response to intravenous estradiol (0.5 μg./100 gm. body weight) have been included in Figure 3. It should be mentioned that the same degree of edema at 4 hours was obtained by introducing only 0.02 μg. (in 0.02 ml.) estradiol-17β into the lumen of the uterus (not shown in Figure).

Exogenous histamine and uterine hydration

Since local liberation of endogenous histamine through the action of Compound 48–80 elicited uterine hyperemia and water imbibition, it was deemed advisable to study the capacity of exogenous histamine to evoke a similar response. Accordingly, varying amounts of histamine dihydrochloride were injected into the lumen of each uterine horn and the tissue assayed for water content 4 hours later. One mg. histamine/horn produced a

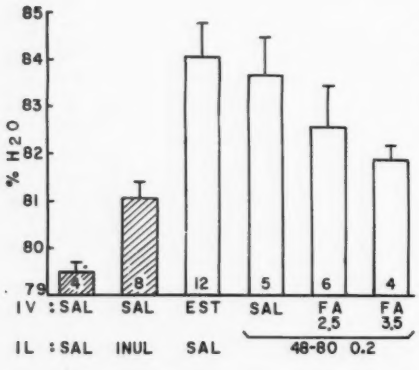

FIG. 3. The effect of cortisol on the uterine hydration due to Compound 48-80. Saline and inulin controls are shown crosshatched. Materials were injected both intravenously (IV) and also directly into the lumen of each uterine horn (IL). Numbers beneath the bars are dosages in mg./100 gm. body weight for cortisol acetate (FA) and mg./horn for 48-80. Saline concentrations employed were 0.9% (IV) or 1.8% (IL); inulin concentration, 2%; estradiol dosage (EST), 0.5 μg./100 gm. body weight. The height of each bar represents the average value resulting from the number of determinations indicated by the enclosed figure. Positive portions of the standard deviations are shown.

response maximum comparable to that induced by intraluminal 48–80 and approaching that elicited by 0.5 μg. of estradiol/100 gm. body weight, I.V. (Fig. 4). Cortisol acetate (2.5 mg./100 gm. body weight, I.V.) given at the same time as the histamine depressed the histamine-induced water uptake to near control levels (Fig. 5).

The influence of antihistamines on estrogen-induced uterine water uptake

A number of pilot studies had indicated that none of the 4 antihistamines tested was effective in altering the uterine response to estrogen when the former were given intravenously or intraperitoneally and under a variety of conditions of pretreatment and dosage. When administered locally into the uterus, however, 2.0–5.0 mg./horn of Chlor-Trimeton (Table 1) or 2.5–5.0 mg./horn of Benadryl were found to be effective in substantially inhibiting water imbibition due to estrogen. The inhibitory action of Chlor-Trimeton was not mediated by increased secretion of anti-inflammatory

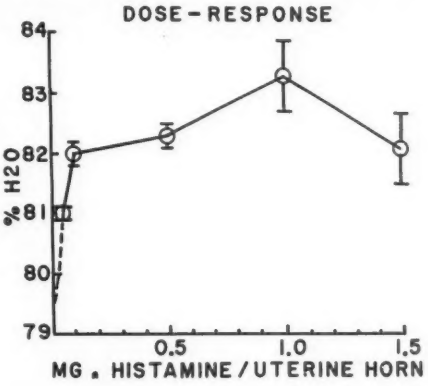

FIG. 4. The effect on uterine water content of increasing amounts of histamine dihydrochloride injected into the lumen of each uterine horn. Each point represents the mean ± the standard deviation of values obtained from 4 animals sacrificed 4 hours after injection.

adrenocorticoids since the antihistamine was equally effective in the absence of the adrenals (Table 1).

Pyribenzamine and Neo-Antergan were ineffective in the above mentioned dosages. The toxicity of these last named compounds was such as to preclude testing in amounts greater than 5.0 mg./horn. The antihistamine experiments involving intrauterine administration over the 2.5–5.0 mg. range are summarized in Figure 6.

Benadryl (I) and Chlor-Trimeton (II), found active in blocking the water response, differ structurally from Pyribenzamine (III) and Neo-Antergan (IV), found ineffective, in that the former group does not contain the intercyclic N atom common to the latter (Fig. 7).

pH of substances administered

At concentrations employed in the experiments, histamine and 48–80 were in the acid range (Table 2). The hyperemia and edema which resulted

TABLE 1. THE INFLUENCE OF CHLOR-TRIMETON MALEATE ON UTERINE WATER IMBIBITION IN ADULT, OVARIECTOMIZED RATS 4 HOURS FOLLOWING ESTROGEN ADMINISTRATION

Experimental conditions	No. of determinations	% uterine water
A. *Saline*—0.5 ml. isotonic/100 gm. body wt., I.V. *Saline*, 0.02 ml. (1.8% instilled into lumen of each horn at same time as I.V. saline.	4	79.5 ± 0.2†
B. *Estradiol-17β*—0.5 μg./100 gm. body wt., I.V. Solution instilled into lumen of each horn at same time as intravenously administered estrogen:		
Saline, 0.02 ml. (1.8%)/horn	12	84.1 ± 0.7
Chlor-Trimeton, 0.5 mg. (0.02 ml.)/horn	4	83.7 ± 0.4
Chlor-Trimeton, 1.0 mg. (0.02 ml.)/horn	4	84.2 ± 0.6
Chlor-Trimeton, 2.0 mg. (0.02 ml.)/horn	5	82.6 ± 0.8*
Chlor-Trimeton, 2.5 mg. (0.02 ml.)/horn	4	82.0 ± 0.9**
Chlor-Trimeton, 5.0 mg. (0.02 ml.)/horn	4	81.4 ± 1.7*
Adrenalectomized just before injections:		
Chlor-Trimeton, 2.5 mg. (0.02 ml.)/horn	4	81.4 ± 0.5**

* $P < 0.01$
** $P < 0.001$ } compared to the I.V. estradiol-intraluminal saline group.
† Mean ± standard deviation.

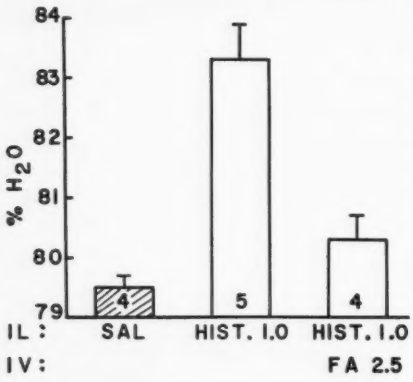

FIG. 5. The influence of intravenous cortisol on the increase in uterine water produced by the intrauterine administration of histamine. Numbers beneath the bars refer to dosage in mg./100 gm. body weight for cortisol acetate (FA) and mg. of the dihydrochloride per uterine horn for histamine (HIST.). Intraluminal saline (SAL) as in Figure 3. The height of each bar is determined by the average value resulting from the number of determinations indicated within the bar. Positive portions of the standard deviations are shown.

from the administration of those compounds cannot, however, be attributed to an acid environment since injection of phthalate-HCl buffer pH 3.1 (Table 2) produced no significant hydration changes from routinely observed control levels in the organ (cf. Figs. 1–6; Table 1). Furthermore, the two most acidic antihistamines were those found to be active in *inhibiting* the water inbibition due to estrogen.

DISCUSSION

Earlier data presented from these laboratories revealed that uterine histamine concentration in the rat is under estrogenic control (1). Depletion of histamine *in situ* was found to be associated with the onset of hyperemia and the imbibition of water in the uterus of the ovariectomized rat treated 4 hours earlier with estradiol-17β. It was further shown that endogenous estrogen secretion in the intact rat was similarly capable of exerting a histamine-liberating influence in the uterus (1).

TABLE 2. pH OF INTRALUMINALLY ADMINISTERED AGENTS

Compound	Concentration, mg. per 0.02 ml.[1]	pH	Effective in depressing uterine H_2O response to estradiol	% uterine H_2O
Pyribenzamine HCl	2.5	6.4	−	
Neo-Antergan maleate	2.5	5.2	−	
Chlor-Trimeton maleate	2.5	5.0	+	
Benadryl HCl	2.5	3.9	+	
Histamine dihydrochloride	1.0	3.8		83.3 ± 0.6[2]
48-80	0.2	3.1		83.7 ± 0.8
Phthalate-HCl buffer	0.02 ml.	3.1		80.6 ± 0.6[3]

[1] Concentration and volume instilled into each uterine horn in experiments described in the text.
[2] Mean ± standard deviation.
[3] $P < 0.001$ (compared to the value for histamine or 48-80).

The present report extends these findings and provides additional support for the view proffered earlier (1) that the acute changes in the vascularity and capillary permeability of the uterus in response to estrogen are mediated by the histamine liberated *in situ* in the presence of the hormone. Histamine or a histamine liberator (Compound 48–80) administered by uterine instillation promotes striking estrogen-like effects in the absence of the hormone. Thus, local vasodilation, hyperemia and water imbibition, characteristic early uterine responses to estrogen action, have been reproduced under these conditions.

These data strongly suggest participation of histamine or related ma-

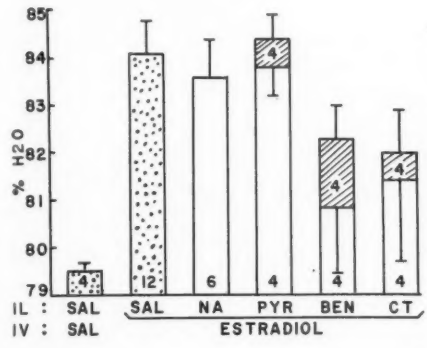

FIG. 6. The influence of antihistaminic compounds on the uterine water response to intravenous estradiol. Antihistamines were given 4 hours before sacrifice directly into the lumen of each uterine horn (IL). Stippled bars represent saline or estradiol controls in dosages and concentrations indicated in Figure 3. In the remaining bars, the total height (clear plus cross-hatched portions) of each represents the effect on uterine water of 2.5 mg./horn of the antihistamine indicated; clear portion only, response to 5.0 mg./horn. Na refers to Neo-Antergan maleate; PYR, Pyribenzamine HCl; BEN, Benadryl HCl; CT, Chlor-Trimeton maleate. The number of determinations for each group appears within each bar. Positive or negative portions of the standard deviations are shown.

terials at a primary stage in the action of estrogen at the target organ. In this connection a possible role for other naturally occurring substances with similar functions should not be excluded from consideration. Serotonin (5-hydroxy-tryptamine), for example, promotes hyperemia and edema formation, especially in the rat, and is depleted from tissues along with histamine by "liberators" of the latter (3, 5).

Isolated findings, suggestive of local histamine mediation in uterine responses to estrogen, have appeared in the literature from time to time. Holden (6) had suggested in 1939 that estrogen action may be mediated by a histamine-like substance when he found that histamine relieved uterine vasoconstriction produced by a variety of agents. Daily parenteral injections of 200 μg. of histamine for 8 days produced in the vagina of the intact rat a histological picture characteristic of pro-estrus and estrus (7). Similar results were reported by Champy, Coujard and Demay (8) on chronic instillation of epinephrine, histamine or choline directly into the

vagina of guinea pigs. Intra-uterine administration of histamine in the rabbit induced morphological changes typical of those elicited by estrogen treatment in the presence of progesterone (9). Given with estrogen over a 5 day period, histamine or acetylcholine enhanced the effect of the steroid in producing an increase in uterine weight (10). Evidence militating against a role for acetylcholine as a natural mediating substance in responses to estrogen has been summarized elsewhere (1, 11).

The blockade of estrogen-stimulated hyperemia and water accumulation

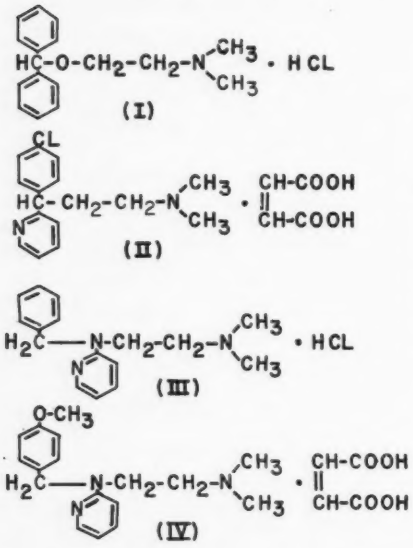

FIG. 7. Structures of the antihistamines used in the studies summarized in Figure 6. Compound I is Benadryl HCl; II, Chlor-Trimeton maleate; III, Pyribenzamine HCl; IV, Neo-Antergan maleate. Compounds I and II were effective in blocking the uterine water response to estradiol whereas III and IV were ineffective in the dosages employed (see text).

by antihistaminic compounds demonstrated in the present studies is consistent with the postulated function for histamine in mediating estrogen action. The significance of the apparent structural requirement for effective antagonism of these early responses to estrogen cannot be stated at this time.

Reports from other laboratories of experiments employing anti-histaminic compounds, support the present hypothesis. Neo-Antergan, given daily for 8 days into the intact rat, resulted in a quiescent vaginal mucosa characteristic of di-estrus-metestrus (7). Though ineffective when either was administered alone, Phenergan and an anti-cholinergic drug injected together prevented estrogen-stimulated estrus in ovariectomized rats (12). Also, Pyribenzamine diminished the potentiating effect of histamine on estrogen in the uterus (10), although that antihistamine was found to be ineffective in blocking the cyclic rhythm of blood flow in endometrial transplants (13).

Other studies have centered about the possible participation of histamine

in the physiology of implantation and pregnancy. Shelesnyak has shown that antihistamines inhibit ovum implantation and the decidual response of pseudopregnancy (14), results also obtained in the rabbit by Chambon and Lefrein (15). Sulman (16) could not confirm the interference with nidation, however, in the mouse. Antihistamines were in addition found by Shelesnyak to interfere with normal pregnancy in the rat and mouse (14), whereas histamine applied topically to the rat uterus actually stimulated deciduomata formation without the necessity of mechanical traumatization (14, see also 9). Shelesnyak has postulated histamine participation in normal ovum implantation and formation of decidua (14). Any hypothesis implicating histamine in the initiation or maintainance of pregnancy, however, must eventually be integrated with the findings that histaminolytic activity was observed to be greatly increased in pseudopregnant uterus (17), in placenta (17, 18), and in plasma (19) and uterus (17) of pregnancy.

The 11-oxy, 17-hydroxycorticosteroids are known inhibitors of estrogen action *in vivo* (20, 21). Szego and Roberts have proposed two alternative mechanisms for this steroidal interaction (11, 21). Of these, competition by the two classes of steroid for sites on protein molecules essential for steroid hormone transport, or at the target organ itself, is supported by *in vitro* data (22, 23). The suggestion was also advanced by these workers that the cortisol-estrogen antagonism exhibited could well have been a reflection of the mutually opposing influences of these steroids on uterine permeability (11, 21, 24).

The present data, while they do not support or refute the competitive inhibition hypothesis, do provide support for the alternative mechanism of antagonism of the two steroids, at the level of some permeability phenomenon involving the uterine vasculature. Results presented elsewhere have shown that cortisol prevented the increases in sodium (25) as well as in fluid which normally occur (20, 21, 25) in response to estradiol alone. In the present studies, intravenously administered cortisol was also seen to diminish uterine hydration produced by local instillation of a histamine releasing agent or of histamine itself. In the light of these observations it would seem reasonable to suggest that cortisol does not interact directly with estrogen under the conditions described. Rather, it appears to do so secondarily by diminishing the vasodilation and accompanying edema attributable to the histamine released by estrogen. Such a mechanism would explain the finding that cortisol when administered concurrently with estrogen did not prevent the estrogen-induced release of histamine, although it did eliminate the other measured responses to estrogen (1). These views are consistent with findings that vasodilation (26, 27) and accompanying increase in capillary permeability (28, 29) and other reactions resulting from histamine liberation or injection in extra-uterine tissues, can be diminished or prevented by the administration of cortisone-like

adrenal steroids (28, 30–35). Thus, cortisol appears to mimic the action of the effective antihistamines studied by antagonizing histamine at its site of action. The present results are therefore in harmony with what is known of the action of 11-oxy, 17-hydroxycorticosteroids in promoting a non-specific increase in capillary resistance (33, 36–38).

It appears increasingly evident that histamine intrinsic to the uterus has one or more functions in the physiology of the pregnant and non-pregnant organ. The extent to which these functions may be interrelated is of fundamental importance. It is not unlikely that the vasodilatory property of histamine, leading to increased permeability of the capillary bed, is basic to all mechanisms involving the amine in the uterus.

Acknowledgments

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CORRELATION OF RADIOIODINE UPTAKE WITH THYROID WEIGHT IN THE MOUSE AT VARYING LEVELS OF EXOGENOUS AND ENDOGENOUS THYROTROPIN STIMULATION[1]

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ABSTRACT

Previous studies have shown that transplanting the pituitary to the anterior ocular chamber in mice alters its ability to stimulate the thyroid gland. Radioiodine metabolism is well maintained whereas thyroid weight falls to the hypophysectomy level. Chronic propylthiouracil treatment in such animals causes a marked increase in thyroidal radioiodine uptake but minimal increase in thyroid weight. In similarly treated normal controls both radioiodine metabolism and thyroid weight are greatly increased. These observations provided further evidence that separate "growth" and "metabolic" thyrotropins might exist, the growth factor requiring direct contiguity of the pituitary with the hypothalamus for adequate secretion.

Since it was possible that radioiodine uptake might be more sensitive to small amounts of a single thyrotropin than thyroid weight, the quantitative response of these two parameters to both exogenous and endogenous thyrotropin was compared. Radioiodine uptake was found to reach a maximum with a considerably smaller dose of TSH than did thyroid weight. The present results indicate that the data previously obtained in mice with heterotopic pituitaries can be explained within the classic framework of a single thyrotropin.

PREVIOUS studies in rats with hypothalamic lesions (1, 2, 3) and mice with heterotopically located pituitaries (4, 5) have indicated that these procedures alter the ability of the hypophysis to stimulate the thyroid gland. Radioiodine metabolism, as indicated by the thyroid/serum (T/S) iodide ratio and thyroidal radioiodine uptake, is well maintained whereas the ability of the thyroid to grow in response to chronic antithyroid drug administration is largely lost.

Investigation of the dichotomy thus produced suggested that there might be two separate thyrotropic factors secreted by the pituitary, one stimulating thyroidal iodine metabolism and one stimulating thyroid growth (2). The original studies in the rat showing this divergence of effects primarily utilized the T/S ratio as an index of iodine metabolism but subsequent work has shown that this parameter is affected to a considerable degree by factors other than TSH (6, 7). In addition, more recent studies

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in the rat have shown that certain hypothalamic lesions depress radioiodine uptake and thyroidal secretion rate as well as thyroid growth (8, 12). Thus evidence is accumulating that it may be unnecessary to postulate the secretion of more than one thyrotropin.

However, the changes produced in thyrotropic activity by transplanting the pituitary to the anterior ocular chamber in mice have not yet been satisfactorily explained on the basis of a single thyrotropin (4, 5). In such animals the thyroid and other target endocrine glands atrophy to the same extent as in completely hypophysectomized mice. In spite of the considerable reduction in thyroid size, the radioiodine uptake and T/S ratio of the animals with heterotopic pituitaries are maintained at an approximately normal level. Chronic propylthiouracil (PTU) administration, which presumably causes a greatly augmented secretion of TSH due to a lowering of circulating thyroxine, will result in augmentation of both the iodide-concentrating mechanism and organic binding of iodine (after stopping PTU for 2 days to allow its excretion). Mice with heterotopic pituitaries under these conditions have a considerably greater absolute level of radioiodine metabolism per unit thyroid weight than do similarly treated normal controls. In contrast, chronic PTU treatment induces a 3–4 fold increase in thyroid weight of normal controls whereas only a slight increase in thyroid weight results from this treatment in mice with transplanted pituitaries.

Although such results were believed best explained by postulating separate "growth" and "metabolic" thyrotropins, the growth factor requiring direct contiguity of the pituitary with the hypothalamus to be elaborated adequately, other explanations were possible (11). The present study was undertaken to see if there might be a difference in the sensitivity of the growth and metabolic responses of the mouse thyroid to both exogenous and endogenous thyrotropin. If the radioiodine uptake were found to have a significantly lower threshold, this would obviate the necessity of assuming the existence of more than one TSH.

MATERIALS AND METHODS

Female Swiss albino mice weighing approximately 20 gm. were used in all experiments. In studying the effect of exogenous thyrotropin, the animals were hypophysectomized by the parapharyngeal route one week prior to beginning the TSH injections. Parke, Davis thyrotropin (lot 50-P4), containing approximately 2 U.S.P. units per mg., was used in all experiments where exogenous TSH was given. Each dose was dissolved in 0.4 ml. physiologic saline and given subcutaneously once daily at rotated sites. To study endogenous thyrotropin secretion, animals were fed a diet containing 0.15% propylthiouracil for three weeks and injected intraperitoneally once daily with graded doses of sodium L-thyroxine dissolved in 0.2 ml. saline. Propylthiouracil was removed from the diet two days before autopsy to allow excretion of the drug. A Remington low iodine diet was fed except where otherwise indicated.

The animals were killed by exsanguination $4\frac{1}{2}$ hours following the intraperitoneal administration of 0.2 μC. I^{131} in 0.2 ml. physiologic saline. The thyroids were removed under

TABLE 1. HYPOPHYSECTOMIZED MICE GIVEN EXOGENOUS TSH

Exp.	Dose of TSH (mg.)	No. of animals	Body weight (gm.)	Thyroid weight		4½ hr. I^{131} uptake		% uptake/mg. thyroid
				(mg.)	% normal controls	% dose	% normal controls	
I	5	2	19.5±2.0	4.11±1.81	138.8±61.1	32.8±6.1	195.2±36.30	9.1±2.5
	2	3	19.3±1.0	4.28±1.00	144.7±33.8	34.2±1.4	203.4± 8.10	8.6±1.7
	0.5	5	19.5±0.6	3.10±0.36	104.6±12.2	21.4±4.8	127.5±28.70	6.8±1.5
	0.2	3	19.3±0.3	2.28±0.31	76.8±10.9	8.6±2.9	51.0±17.50	3.6±0.9
	0	4	18.0±1.5	1.78±0.27	60.2± 9.1	0.4±0.1	2.4± 0.50	0.2±0.0
	Normal Controls	7	25.0±1.5	2.96±0.34	100.0±10.0	16.8±1.1	100.0± 1.40	6.2±1.3
II	5	4	17.3±0.5	3.33±0.80	142.4±34.3	42.8±6.3	147.8±21.60	13.7±2.0
	2	5	17.9±0.8	2.10±0.24	89.7±10.3	21.4±4.9	73.9±16.80	10.2±1.9
	0.5	8	17.9±0.5	2.09±0.12	89.4± 5.1	21.9±1.9	75.5± 6.40	10.5±0.7
	0.2	7	17.5±0.5	1.64±0.09	69.9± 3.8	14.9±4.9	50.9±16.60	8.6±2.2
	0	4	16.3±0.6	1.92±0.26	82.1±11.0	0.5±0.1	1.5± 0.23	0.3±0.1
	Normal Controls	6	21.6±0.5	2.34±0.28	100.0±12.0	29.0±2.6	100.0± 8.90	13.6±2.5

Results expressed as mean and standard error. Indicated dose of TSH given once daily for 7 days, animals autopsied on 8th day.

a 6× dissecting scope and weighed on a Sartorious torsion microbalance with a sensitivity of 0.01 mg. Following weighing, the glands were placed in test tubes containing 2 ml. of water and counted in a scintillation well counter having a sensitivity of 10^6 cpm/μC. Completeness of hypophysectomy was checked by examination of the sella under 10× magnification at autopsy. Any animals with questionable pituitary remnants were discarded.

RESULTS

A. *Exogenous Thyrotropin:* The results are summarized in Table 1 and Figure 1. As has often been noted previously, hypophysectomy resulted in a much greater decline in radioiodine uptake than in thyroid weight. Unfortunately, because of our limited supply of thyrotropin and the insensi-

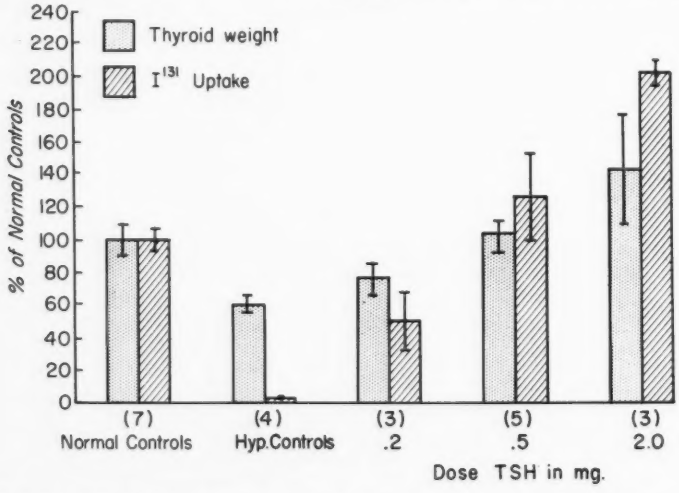

FIG. 1. TSH given to hypophysectomized mice. Graphic plot of data in Experiment I. Figures in parentheses refer to number of animals. Since only 2 mice survived in the group receiving 5 mg. TSH, this group was not plotted.

tivity of the thyroidal response, it was not possible to obtain a maximal effect from even the largest dose of thyrotropin given. Even with the submaximal response, however, it is apparent that a steeper dose-response slope was obtained with radioiodine uptake than with thyroid weight.

B. *Endogenous Thyrotropin:* The results are summarized in Table 2 and Figure 2. By using varying doses of thyroxine in combination with amounts of propylthiouracil which would assure as complete inhibition of thyroxine synthesis as could be obtained, it was possible to control the rate of secretion of endogenous thyrotropin over as wide a range as desired. The steeper dose-response slope of radioiodine uptake obtained with exogenous thyrotropin was confirmed with endogenous TSH. In addition, it was found that with the faster rate of increase of the radioiodine uptake this parameter reached a plateau with a dose of thyroxine at least 3 times as large as that used when a plateau in thyroid weight was reached. This indicated that a much smaller amount of circulating thyrotropin caused maximal radioiodine uptake than was required to produce maximal thyroid weight.

It is of interest that an "overshoot" was produced at the beginning of the radioiodine uptake plateau (Fig. 2). Once the dose of thyroxine had been reduced to a point where the uptake had increased sufficiently to equal that of the PTU controls, a further reduction in thyroxine dosage would consistently result in an increase in radioiodine uptake above that seen in the PTU controls. As the dose of thyroxine was reduced to zero, the

TABLE 2. PTU-FED MICE GIVEN GRADED DOSES OF THYROXINE

Exp.	Dose of thyroxin	No. of animals	Body weight (mg.)	Thyroid weight		4½ hr. I^{131} uptake		% uptake/mg. thyroid
				(mg.)	% PTU cont.	% Dose	% PTU cont.	
III	10	7	20.6±1.72	1.83±0.11	20.0± 1.20	0.47±0.09	1.7± 0.50	0.26±0.08
	5	7	20.9±1.29	2.17±0.20	23.7± 2.20	0.50±0.04	1.8± 0.15	0.24±0.03
	2	7	21.9±0.86	2.39±0.51	27.7± 5.30	9.40±4.80	32.9±16.70	2.87±0.93
	0.5	7	21.6±1.28	7.00±0.98	76.2±10.70	35.60±4.90	124.5±16.90	6.28±1.71
	0	7	22.4±0.71	9.15±0.69	100.0± 0.13	28.60±1.56	100.0± 0.42	3.24±0.29
	Purina Controls	5	24.9±1.70	1.98±0.13	21.6± 1.40	2.20±0.42	7.7± 1.40	1.12±0.23
	L.I. Controls	6	21.7±1.33	2.99±0.88	32.7± 9.60	23.40±5.50	81.9±19.20	10.43±3.85
IV	5	8	24.0±0.44	2.43±0.11	21.5± 1.00	3.90±0.90	6.4± 1.50	1.60±0.31
	2	8	21.3±1.00	4.53±0.74	40.1± 6.50	38.30±8.20	62.7±13.40	8.45±2.40
	1	7	21.6±0.79	10.50±0.62	92.8± 5.50	85.70±1.10	140.3± 1.70	8.16±0.48
	0.7	8	22.6±0.69	11.92±0.87	105.4± 7.70	82.50±2.40	135.0± 4.00	6.92±0.66
	0.5	8	21.0±0.81	12.59±1.40	111.4±12.50	65.80±4.60	107.7± 7.50	5.23±0.45
	0.2	8	20.8±0.50	10.70±0.97	94.6± 8.60	61.50±5.40	100.7± 8.80	6.26±1.23
	0	8	20.9±0.56	11.31±1.10	100.0± 0.83	61.10±2.10	100.0± 2.13	5.40±0.32
	L.I. Controls	8	22.8±0.81	3.57±0.30	31.6± 0.50	53.90±2.80	88.2± 4.60	15.10±1.27
V	5	9	19.2±0.38	1.83±0.10	23.2± 1.30	2.40±0.40	4.3± 0.80	01.40±0.30
	3	11	19.4±0.50	2.38±0.37	30.2± 4.70	15.80±4.50	28.0± 7.90	05.60±1.20
	2	11	20.0±0.33	3.34±0.28	42.5± 3.40	49.80±6.40	88.1±10.40	13.80±1.60
	1.5	10	17.9±0.75	4.27±0.64	54.2± 8.10	61.20±8.70	108.5±13.60	15.30±2.60
	1	10	18.3±0.60	4.84±0.46	61.5± 5.90	63.50±6.40	112.6±11.00	13.50±1.30
	0.7	7	20.7±0.50	5.81±0.65	73.8± 8.30	63.80±5.70	113.1±10.20	09.10±0.80
	0.5	9	19.3±0.50	7.21±0.64	91.6±10.00	58.80±3.50	104.3± 6.30	09.00±1.30
	0	8	19.1±0.68	7.87±0.69	100.0± 8.80	56.40±2.70	100.0± 4.80	07.80±0.70

Results expressed as mean and standard error. Indicated dose of thyroxine given daily. Purina controls were fed Purina fox checkers. All groups were fed propylthiouracil in the diet except the Purina controls and low iodine controls. All animals except the Purina controls were fed a low iodine diet. PTU Cont. =animals fed the low iodine, PTU die but did not receive thyroxine. L.I. Controls =animals fed low iodine diet. Neither the Purina nor low iodine control groups received thyroxine.

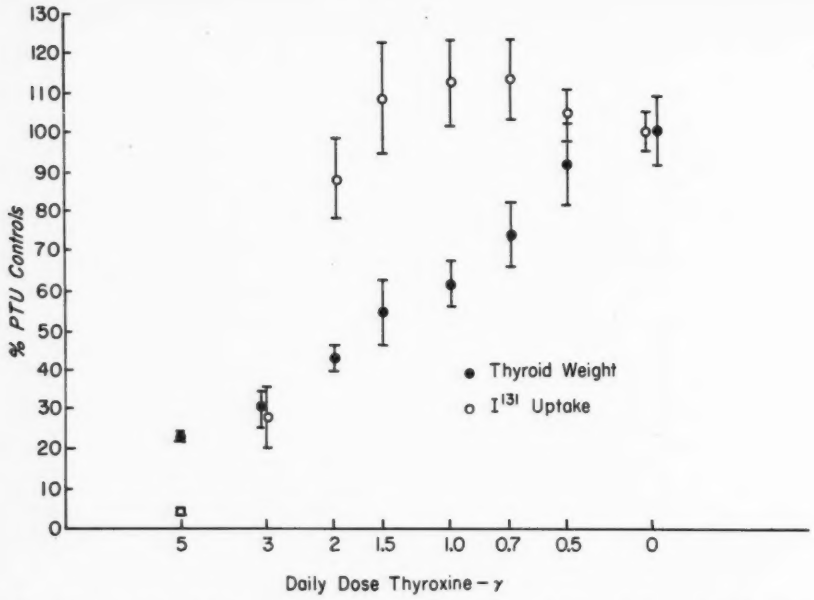

FIG. 2. Thyroid weight vs. uptake in PTU-fed mice administered thyroxine. Graphic plot of data in Experiment V.

iodine uptake would fall to the same level observed in the PTU controls. The height of the overshoot was maximal at a daily dose of 0.7–1.0 μg. thyroxine.

DISCUSSION

The results obtained in these experiments support the concept that the high radioiodine uptake with little thyroid growth response exhibited by mice with heterotopic pituitaries may be due to a greater sensitivity of thyroidal radioiodine metabolism than thyroid growth to small amounts of thyrotropin. If both growth and iodine metabolism were equally sensitive, one would expect that both parameters would reach a plateau at the same level of thyrotropin. If one were more sensitive, its plateau would be expected to begin at a smaller dose. The data recorded here clearly indicate that a plateau was first reached with radioiodine uptake.

In some of the earlier experiments in mice with heterotopic pituitaries (5), chronic propylthiouracil treatment produced an absolute increase in the radioiodine uptake per unit thyroid weight of such animals which was 4-fold greater than that produced in similarly treated normal controls. Failure to achieve this level in the present experiment is more likely due to to individual variations in the animals than to a qualitative difference in the response. Not all groups of mice with pituitary transplants previously

studied (5) achieved a 4-fold greater uptake per unit thyroid weight than their controls. It is of interest that in the present experiments the highest I^{131} uptake/mg. thyroid occurred in animals receiving low iodine diet alone.

The "overshoot" of the radioiodine uptake with increasing amounts of endogenous TSH was seen consistently in all experiments, although its significance is not clear at this time. It possibly suggests further the negative feedback control system by which the level of circulating thyroxine regulates TSH output, overshoots of this nature being characteristic of "servo" mechanisms. Another possible explanation is that a small amount of thyroxine may enable the pituitary to secrete more TSH than it does in the complete absence of thyroxine. Such a concept is supported by previous evidence in human myxedema where the urinary or blood levels of TSH are transiently increased after the initiation of therapy with thyroid hormone (9, 10).

Our interpretation of the present results is that they add to a growing body of evidence suggesting that the apparent divergence of various parameters of thyroid function under certain conditions may more likely be explained by different thresholds of the individual processes to a single thyrotropin than that more than one thyrotropin is involved in their control. It is worth noting, however, that Florsheim (8) has recently found that thyroidal radioiodine uptake and protein-bound iodine can be suppressed in the rat by hypothalamic lesions which do not prevent thyroid hypertrophy due to administration of antithyroid drugs. Since this is the converse of the present findings, the possible existence of more than one thyrotropin has not yet been finally excluded.

Acknowledgment

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FUNCTIONAL ZONATION OF THE ADRENAL CORTEX: PATHWAYS OF CORTICOSTEROID BIOGENESIS

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ABSTRACT

Direct proof that aldosterone is produced by the glomerulosa of beef and rat adrenals has recently been provided. In the present investigation, the functional zonation of beef adrenal cortex has been further studied *in vitro*.

When progesterone, desoxycorticosterone and corticosterone are added to the incubation media of beef adrenal slices they are metabolized in radically different ways depending on whether the slices originate in the glomerulosa (G) or in the fasciculata-reticularis (F.R.). In the presence of tissue taken from G these steroids increase the production of aldosterone.

Desoxycorticosterone increases the formation of corticosterone both in G and F.R. Progesterone enhances the production of hydrocortisone in F.R. only, whereas it increases corticosterone and aldosterone production in G. 21-hydroxypregnenolone increases the production of aldosterone in G and that of corticosterone in both G and F.R. 17α-hydroxyprogesterone and 17α-hydroxy-11-desoxycorticosterone markedly enhance the production of hydrocortisone in F.R. and lead to the formation of this compound in G where normally it is not formed. Progesterone, desoxycorticosterone and corticosterone increase the production of aldosterone by G to the same extent.

These findings provide indirect evidence for an enzymatic specificity within the different types of cortical cells which is responsible for the anabolism of different types of hormones from a possibly identical precursor. Thus, the evidence tends to indicate the occurrence of 18-oxygenation in G but not in F.R., of 17-hydroxylase activity in F.R. but not in G, and that of 3β-dehydrogenase activity and 11- and 21-hydroxylase activities in both.

INTEREST in the functional specificity of the different cellular zones of of the mammalian adrenal cortex arose from the inspiring investigations of Deane, Greep and collaborators (1, 2, 3) who first correlated morphological changes of the adrenal cortex with changes in mineral balance, pituitary function, and carbohydrate metabolism.

Although their conclusions were confirmed by a number of workers the validity of their methods, as well as the interpretation of their results, has been debated. Following extensive studies of mice adrenal cortex, Chester Jones (4) concluded that in eutherian mammals the existence of the glomerulosa is only due to the presence of a poorly elastic capsule of connective tissue and that this zone represents a "vegetative back-water" of cortical

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cells. However, the recent demonstration that aldosterone is exclusively produced by the zona glomerulosa of beef and rat adrenals (5–8) has lent great support to the conclusion of Deane, Greep and their group.

The aim of the following investigation is to provide further evidence for the concept of "functional zonation" by demonstrating the existence of different pathways of corticosteroid biogenesis within the different cellular layers of the adrenal cortex.

METHODS

Incubation

Beef adrenal glands were collected at the slaughterhouse within the hour following the death of the animals. The glands were dissected free of peripheral fat and bisected. The medulla was discarded. The cortex, with the remaining connective tissue of the capsule, was cut into slices 1–1.5 cm. wide and 0.3–0.5 mm. thick. The zona glomerulosa was separated from the two inner cortical zones by cutting with a razor blade along a line of pigmented cells, visible to the naked eye, which was histologically proven to correspond with the innermost cellular layers of the glomerulosa (see Figure 1). The slices were then transferred into the compartments of a partitioned Petri dish, in a solution of 0.9% sodium chloride. In each series of experiments, the glomerulosa slices and their fasciculata-reticularis counterparts were evenly distributed among the different groups of tissue to be incubated so that equally representative parts of the cortex could contribute to each group of tissue. From the time of collection at the slaughterhouse until the beginning of the incubation, an average period of three hours, the tissues were kept in a solution of 0.9% sodium chloride at 5 to 8° C—except for the time necessary to prepare the slices. Prior to incubation, the slices were decanted, gently blotted on filter paper to absorb excess saline, and weighed on a torsion balance. Each group of slices was then transferred to a 50 cc. beaker containing 10 cc. of incubation medium per gram of tissue. The slices were incubated in Krebs Ringer Bicarbonate medium with added glucose (200 mg.%) (9, 10). This medium was always freshly prepared from concentrated mother solutions kept at 5° C and renewed every 4 months. The incubation was performed in a Dubnoff Metabolic Shaker Incubator in an atmosphere of oxygen—5% CO_2, at 37 ± 1° C. The flow of gas and the shaking were regulated at 5 liters/minute, and 100 movements/minute, respectively. According to the method recommended by Saffran (10), the slices were pre-incubated for a period of half an hour, at the end of which the medium was renewed and the incubation (per se) allowed to proceed for 2 hours. When corticosteroids precursors were used, chosen amounts of these compounds, kept in methanol solution, were pipetted into a set of clean beakers. The methanol was evaporated off under a stream of nitrogen. The steroids were redissolved in the Krebs Ringer Bicarbonate medium which, if necessary, was slightly warmed to hasten the solubilization. The pre-incubated slices were then transferred to these beakers. At the end of the incubation, the media were collected and the slices rinsed a few times with small portions of distilled water. The rinsings were added to the respective incubation media. These were frozen until extraction. In most experiments 1.5–2.0 gm. of glomerulosa and 3.0–4.0 gm. of fasciculata-reticularis were used to make up individual groups of slices but up to three times these amounts were needed when the incubation was performed in the absence of added steroid precursors.

Extraction

The media were extracted first with twice, and then with once their volume of chilled, redistilled chloroform. Emulsions were broken down by centrifugation. The chloroform

extracts were pooled, filtered into round-bottom flasks so as to absorb residual traces of water, and evaporated to 2 cc. under vacuum at a water bath temperature of $50 \pm 2°$ C. The concentrated extract was dried under nitrogen. In each set of experiments an appropriate volume of incubation medium was extracted to be used eventually as a "blank."

Crude Extracts

In a number of experiments, aliquots of the crude lipid extracts were taken for quantitative analysis of total Δ^4-3 ketonic steroids at 2400 Å (10), of total α-ketolic reducing steroids by the micro blue tetrazolium reaction of Genest (11) and of dihydroxyacetone steroids by the Porter-Silber reaction (12). These determinations were performed against proper aliquots of the incubation medium "blank" carried through the entire procedure. In most cases 1/20 of the crude lipid extract of each incubation medium was sufficient for each of these determinations.

Chromatography

All chromatographic developments were done at room temperature ($25 \pm 2°$ C), after overnight equilibration for the system of Bush (13), and after 12 hours equilibration at 35° C, in a constant temperature cabinet for the system E^2B of Eberlein Bongiovanni (14). Detection of the steroids on the chromatograms was achieved by direct visualization and/or contact photography under ultraviolet light. If elution of the steroids was not performed, the combined blue or triphenyltetrazolium NaOH fluorescence reaction (15) was developed on the paper. The crude lipid extracts were in all cases submitted to a first chromatography in the system B5 of Bush (benzene-methanol-water, 2:1:1) over a period of 4 to 5.5 hours.

Partial Identification

Partial identification of aldosterone, hydrocortisone, and corticosterone was achieved by assessing their respective chromatographic mobilities in the system propyleneglycol: toluene of Zaffaroni (16), in the system C of Bush and in the system E^2B of Eberlein Bongiovanni; by mixed chromatography with the corresponding crystalline compound and by the identity of their respective sulfuric acid spectra with that of pure compounds (15). Aldosterone was furthermore characterized by its biological activity in either the Venning (17) or the Simpson-Tait (18) assay. Aldosterone, hydrocortisone and corticosterone were subjected at least once to this complete series of tests in each group of experiments, but not in individual experiments where the conditions of incubation and the precursor studied remained unchanged.

Spectrophotometric Determination

The quantitative determination of Δ^4-3 ketosteroids was carried out at 2400 Å according to the method described by Saffran (10). Spectrophotometric measurements of these steroids were performed in methanol solution against the eluate of a similar area of the paper on which the extract of the incubation medium "blank" had been chromatographed. Hydrocortisone and corticosterone were quantitatively measured by this method after one single chromatography in the system B5; aldosterone after a second chromatography in the system C of Bush. A greater chromatographic purification of aldosterone was not considered essential for the following reasons:

1) after two successive chromatographic developments in the systems B5 and C aldosterone if reapplied on the iso-octane tert-butanol aqueous methanol system (14) behaves as homogeneous Δ^4-3 ketone material.
2) the values of aldosterone obtained by this method are very close to those obtained by bioassay.

RESULTS

In a preliminary experiment an attempt was made to incubate separately the three zones of beef adrenal cortex. Consequently, after the separation of the zona glomerulosa the innermost fourth of the fasciculata-reticularis slices was separated. This innermost portion of the cortex is designated here zona reticularis. Although in beef adrenal cortex the existence of a well-defined zona reticularis is not unanimously recognized, the concept of certain authors considering the innermost part of the cortex to be a zone of cellular degenerescence made it of interest to investigate its ability to produce hormones upon incubation. The quantitative estimation of Δ^4-3 ketosteroids, α-ketolic and 17-hydroxycorticosteroids produced on incubation by the glomerulosa, the fasciculata, and the reticularis of beef adrenal cortex is presented in Table 1 together with the total sodium-retaining activity of the crude lipid extracts of their respective incubation media

From this experiment it appeared that 17-hydroxycorticosteroids (Porter-Silber chromogens) are produced almost entirely by the fasciculata and the reticularis. Assuming that the beef adrenal cortex produces negligible amounts of 17-hydroxycorticosteroids reduced in ring A, one is led to conclude that by far the largest portion of Δ^4-3 ketosteroids produced by the fasciculata and the reticularis bears a dihydroxyacetone side chain, whereas Δ^4-3 ketosteroids bearing an α-ketolic side chain originate mostly in the glomerulosa. The ratio of Porter-Silber chromogens/Δ^4-3 ketoster-

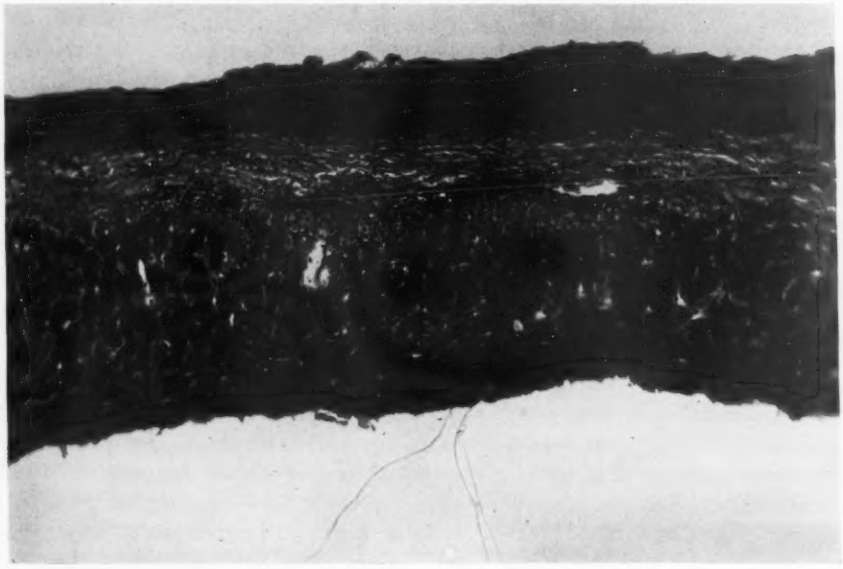

FIG. 1. Microphotograph of glomerulosa slice (×85; Masson Trichrome). Connective tissue of the capsule is seen adhering to zona glomerulosa.

TABLE 1. COMPARISON OF THE STEROID MATERIAL PRODUCED ON INCUBATION BY THE DIFFERENT CELLULAR ZONES OF BEEF ADRENAL CORTEX

	μg./100 gm. of tissue/hour			
	U.V.[1]	B.T.Z.[2]	P.S.[3]	M.A.[4]
Glomerulosa	1030	750	20	130
Fasciculata	450	375	270	4
Reticularis	400	270	180	0

[1] Maximal absorption at 2400 Å.
[2] Blue Tetrazolium reaction.
[3] Porter-Silber reaction.
[4] Mineral-corticoid activity in the Simpson-Taits assay (18) expressed in μg. of aldosterone.

oids is equal to .02 in the glomerulosa as opposed to 0.6 and 0.45 in the fasciculata and the reticularis respectively.

The table also shows that a certain proportion of steroids possessing a Δ^4-3 ketone group but no 17-α-ketolic side chain is produced in the three cortical zones, possibly in slightly greater amounts by the zona glomerulosa.

Finally, it is most striking to note that practically all the sodium-retaining activity is found in the crude extract of the incubation medium of glomerulosa slices, whereas it is negligible in the incubation media of the fasciculata and the reticularis. Following this experiment, it was not again attempted to incubate the zona reticularis separately.

After paper chromatography, the main compounds produced by incubation of glomerulosa slices were identified as aldosterone and corticosterone; those produced by the zona fasciculata-reticularis as hydrocortisone and corticosterone (see Figure 2).

No aldosterone was detected on the chromatograms of the incubation media of fasciculata-reticularis, and only traces of hydrocortisone on the chromatograms of the incubation media of glomerulosa. It is highly probable that these traces of hydrocortisone, which in all cases were too small to be measured by spectrophotometry, were produced by clusters of fasciculata cells incompletely separated from the zona glomerulosa during dissection of the tissue.

Two unidentified ultraviolet absorbing substances which did not reduce tetrazolium salts were found to be regularly produced in the incubation media of glomerulosa slices. In the system B5 of Bush their chromatographic mobilities were respectively slower than that of the hydrocortisone pilot and slightly faster than that of the cortisone pilot. Besides hydrocortisone and corticosterone two Δ^4-3 ketone, α-ketolic compounds were consistently observed after chromatography of the extracts of the incubation media of fasciculata-reticularis slices. In the system B5 of Bush, one was more polar than hydrocortisone, the second had a polarity very similar to the pilot of cortisone. These substances are not homogenous and can be

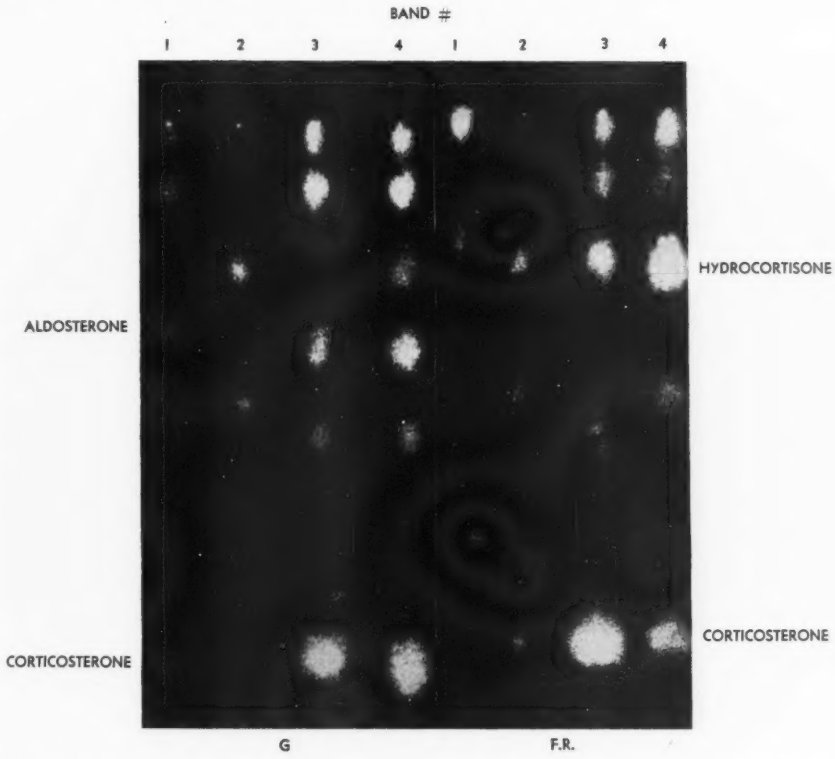

FIG. 2. U.V. photostat of chromatograms from the system B5 of Bush on which extracts of incubation media of glomerulosa (G) and fasciculata-reticularis (F.R.) slices were developed. Band 1: "Control"; Band 2: Pilots Hydrocortisone, Cortisone and Corticosterone: 10 μg. of each; Band 3: incubation with desoxycorticosterone; Band 4: incubation with progesterone.

resolved into two or three compounds by further chromatography. Finally, a steroid, partly identified as 11-dehydrocorticosterone, was recognized as being produced by both parts of the cortex.

The lowest part of Table 2 shows that the basic production of aldosterone by glomerulosa slices is equal to 37 ± 5 μg./100 gm. of tissue/hour; that of hydrocortisone by the fasciculata-reticularis to 61 ± 6 μg./100 gm. of tissue/hour. Corticosterone is produced at the rate of 58 ± 8 and 45 ± 9 μg./100 gm. of tissue/hour by glomerulosa and fasciculata-reticularis slices, respectively. When known precursors of corticosteroids such as desoxycorticosterone, progesterone, and corticosterone were added to the incubation media at the dose level of 50 μg./gm. of tissue/2 hours of incubation, they were handled in radically different ways depending on whether

TABLE 2. PRODUCTION OF ALDOSTERONE, HYDROCORTISONE AND CORTICOSTERONE BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL INCUBATED WITH DESOXYCORTICOSTERONE (DOC), PROGESTERONE AND CORTICOSTERONE (B)

Steroid added[1]	Glomerulosa				Fasciculata-Reticularis			
	Tissue weight incubated (mg.)	μg./100 gm. tissue/hour			Tissue weight incubated (mg.)	μg./100 gm. tissue/hour		
		Aldosterone	Hydrocortisone	Corticosterone		Aldosterone	Hydrocortisone	Corticosterone
DOC	2372	82	traces	290	3709	Nil	150	350
	1824	109	traces	576	3360	Nil	30	120
	1898	75	traces	130	2852	Nil	78	280
	1892	127	traces	500	2882	Nil	70	340
Progesterone	2355	79	traces	250	3615	Nil	380	140
	1985	115	traces	75	3898	Nil	320	60
	1761	134	traces	298	3210	Nil	155	62
	1962	133	traces	490	3010	Nil	255	180
B	1795	115	traces	Not measured	3486	Nil	60	Not measured
	1674	220	traces	Not measured	2944	Nil	60	Not measured
	1976	142	traces	Not measured	3900	Nil	59	Not measured
Mean Control production μg./100 gm. tissue/hr.		37 ± 5*	traces	58 ± 8		Nil	61 ± 6	45 ± 9

[1] 5 μg./100 mg. of tissue/2 hours of incubation.
* Standard Error of the mean.

the slices originated in the glomerulosa or in the fasciculata-reticularis (see Table 2 and Figure 2). In the presence of glomerulosa slices, these three steroids increased the production of aldosterone, but they did not lead to the formation of this hormone by the fasciculata-reticularis. Only progesterone enhanced the production of hydrocortisone, and this only in the incubation media of fasciculata-reticularis slices. The addition of progesterone, desoxycorticosterone, and corticosterone to the incubation media of glomerulosa slices did not promote the formation of hydrocortisone which, as in the control, was found only in negligible amounts. However, if important fragments of fasciculata remained attached to the glomerulosa, measurable amounts of hydrocortisone were found in the incubation media of glomerulosa slices after incubation with progesterone.

In both the glomerulosa and the fasciculata-reticularis, desoxycorticosterone markedly increased the production of corticosterone; whereas progesterone enhanced the production of this steroid mainly in the zona glomerulosa. On the other hand, 17-hydroxylated steroid precursors such as 17-hydroxyprogesterone and 17-hydroxy-11-desoxycorticosterone led to the formation of large amounts of hydrocortisone, not only in the fasciculata-reticularis, but also in the glomerulosa (see Table 3).

It can be calculated that in the presence of glomerulosa slices, these steroids have been converted to hydrocortisone to an extent of 60%. This observation demonstrates the efficiency of 21- and 11-hydroxylations on a 17-hydroxylated precursor incubated with glomerulosa tissue.

In order to study the capacity of the glomerulosa and the fasciculata-reticularis to promote the transformation of the Δ^5-3 ol to the Δ^4-3 ketone structure, it was decided to incubate pregnenolone in the presence of these two parts of the cortex. However, since pregnenolone was not available, its

TABLE 3. PRODUCTION OF HYDROCORTISONE (F) BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL INCUBATED WITH 11-DEHYDRO-17-HYDROXY-CORTICOSTERONE (S) AND 17-HYDROXY-PROGESTERONE (17-OH-PROG.)

Steroid added μg./100 mg. of tissue	Tissue weight incubated (mg.)	Glomerulosa (F: μg./100 gm. tissue/hr.)	Tissue weight incubated (mg.)	Fasciculata-reticularis (F: μg./100 gm. tissue/hr.)
S: 10 μg.	1540	1460	3204	1120
S: 10 μg.	1540	1900	3204	1030
S: 10 μg.	1425	1400	1680	1710
S: 10 μg.	1425	1770	1680	2900
17-OH-Prog. 5 μg.	1518	490	1835	810
17-OH-Prog. 5 μg.	1518	540	1835	580
17-OH-Prog. 10 μg.	1500	2100	3176	780
17-OH-Prog. 10 μg.	1500	2670	3176	590
Mean Production of Hydrocortisone by Controls μg./100 gm./hour		traces		55 ± 3.8[1]

[1] Standard Error of the mean: 8 experiments.

21-hydroxylated derivative (at the dose of 100 μg./gm. of tissue/4 hours of incubation) was used in the experiment reported in Table 4. 21-hydroxypregnenolone increased the production of aldosterone by the zona glomerulosa and markedly enhanced the production of corticosterone by both parts of the cortex. The production of hydrocortisone by the fasciculata-reticularis was also increased but to a lesser extent. This may reflect a limited ability of 17-hydroxylation on a 21-hydroxylated substrate.

To investigate further the pathway of aldosterone biogenesis, glomerulosa slices were incubated in the presence of increasing amounts of progesterone, desoxycorticosterone and corticosterone. Each of these steroids was added to the incubation media at the dose levels of 5, 10 and 20 mg./100 gm. of glomerulosa tissue. The period of incubation lasted 2 hours. The results of these experiments are summarized in Figure 3. They show that the production of aldosterone/unit of tissue weight/hour was proportional to the amount of steroid added, and that at a given dose these three steroids increased the production of aldosterone above that of the controls in

TABLE 4. PRODUCTION OF ALDOSTERONE, HYDROCORTISONE AND CORTICOSTERONE BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL INCUBATED WITH 21-HYDROXYPREGNENOLONE

Steroid added[1]	Glomerulosa				Fasciculata-Reticularis			
	Tissue weight incubated (mg.)	μg./100 gm. tissue/hour			Tissue weight incubated (mg.)	μg./100 gm. tissue/hour		
		Aldosterone	Hydrocortisone	Corticosterone		Aldosterone	Hydrocortisone	Corticosterone
21-OH Pregnenolone	1320	240	traces	393	2468	Nil	210	250
	1338	299	traces	239	2608	Nil	144	138
	1356	310	traces	200	2395	Nil	117	200
Control #1	1600	99	Nil	18	3086	Nil	47	18
Control #2	1500	86	Nil	26	2614	Nil	55	16

[1] 10 μg. of 21-OH-Pregnenolone/100 mg. of tissue/4 hours of incubation.

strictly comparable proportions. For instance the amounts of aldosterone recovered after incubation with progesterone, desoxycorticosterone and corticosterone were respectively equal to 250 ± 37 (S.D.), 253 ± 39, and 250 ± 40 μg./100 gm. of tissue/hour when these steroids were added to the incubation media at the dose of 10 mg./100 gm. of glomerulosa slices. These figures represent a mean increase of almost 600% above the mean production of the controls (37 ± 10), and a conversion of 5% of the added steroids to aldosterone over the 2 hours of incubation.

Regarding the 18-oxygenation occurring only in the glomerulosa it may

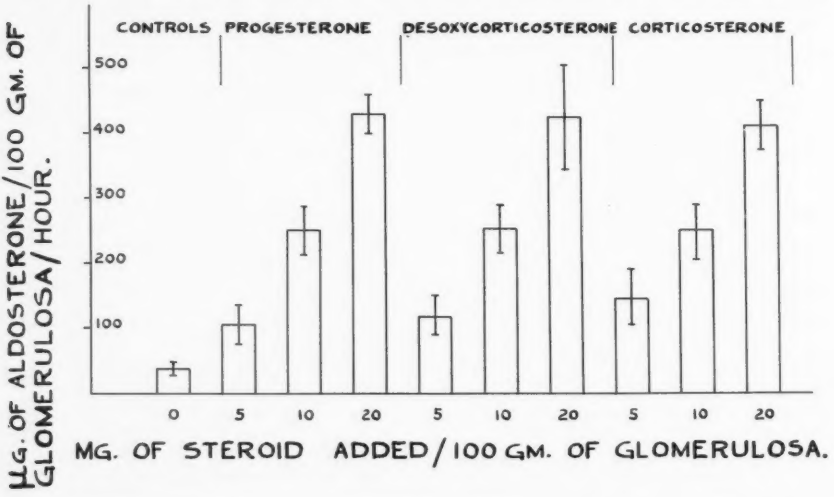

FIG. 3. Production of aldosterone by the zona glomerulosa of beef adrenal incubated with progesterone, desoxycorticosterone and corticosterone ordinate is μg. of aldosterone produced on incubation per 100 gm. of glomerulosa/hour when the tissue was incubated in the presence of increasing doses of progesterone, desoxycorticosterone and corticosterone. The vertical lines represent the magnitude of the standard deviations of the mean.

be of interest to mention the following observation. When 11-dehydrocorticosterone and 11 α-hydroxyprogesterone were incubated with the glomerulosa, two compounds were obtained possessing a chromatographic mobility practically identical with that of aldosterone in the systems B5 and C of Bush and E^2B of Eberlein-Bongiovanni. These two compounds were shown to possess a maximal absorption at 2400 Å in methanol solution and to give a typically positive soda fluorescence reaction; but they neither reduced blue tetrazolium salt nor did they present sodium-retaining activity on bioassay. Since these compounds were essentially formed in the glomerulosa, it is tempting to credit them both with an oxygen function on position 18. One could therefore speculate that 11-dehydrocorticosterone would give rise to 11-dehydrocorticosterone 18-al; 11 α-hydroxyprogester-

one to 11-epialdosterone or to 11-dehydrocorticosterone 18-al. The preferential formation of 11-dehydrocorticosterone 18-al could find support in the finding of large amounts of 11-dehydrocorticosterone in the incubation media of glomerulosa slices incubated in the presence of 11α-hydroxyprogesterone in accordance with the original finding of Zaffaroni (19) in beef adrenal homogenates. In either case the formation of aldosterone hemiacetal not being possible, there could be interaction between the 18-aldehyde and the α-ketolic side chain which would explain the lack of reducing properties of the latter.

DISCUSSION

The concept of the functional zonation of the adrenal cortex arose primarily from Swan's hypothesis (20) which implies that the zona glomerulosa is responsible for the regulation of electrolyte metabolism and is little affected by hypophysectomy whereas the fasciculata-reticularis, highly dependent on the presence of the anterior pituitary, is the site of formation of carbohydrate-regulating hormones. This view was, for the first time, put to the experimental test by Sarason (21) who demonstrated a regression of the zona glomerulosa in rats receiving desoxycorticosterone and later by Deane, Greep and their collaborators who fully confirmed and extended this experimental finding by correlating histochemical changes in the glomerulosa and the fasciculata-reticularis of rat adrenals with induced changes in carbohydrate and electrolyte metabolism (1, 2, 3).

The recognition of aldosterone as the chief mineralocorticoid evoked the interest of various investigators in the possible site of its production within the adrenal cortex, until different groups of workers provided direct proof of its elective production by the zona glomerulosa (5, 6, 7, 8).

The present experiments confirm the original findings of Ayres, Gould, Simpson, and Tait on the production of aldosterone by the glomerulosa, of hydrocortisone by the fasciculata, and of corticosterone by both layers of the ox adrenal gland (5). They indicate further that practically all the biologically active substances controlling electrolyte metabolism (sodium-retaining substances) are produced by the zona glomerulosa, but do not support the conception that the innermost cellular layers of the cortex are formed of degenerating or dying cells. This last assertion is based on the observation that, on incubation, cortical slices from the innermost layer of the cortex produced steroid materials similar to those produced by the fasciculata.

The present investigation provides strong evidence for the existence of different pathways of corticosteroid biogenesis within different parts of the cortex. Its significance lies mostly in the observation that in the presence of an identical steroid precursor, a definite kind of cell is specifically and exclusively able to achieve the whole metabolic transformation necessary for

the formation of specific hormones recognized in a number of animal species, including man, as being the chief products of adrenocortical secretion. For example, progesterone, through 17, 21 and 11-hydroxylations (22), leads to the formation of hydrocortisone only by the fasciculata-reticularis and, through 21-, 11-hydroxylations, and 18-oxygenation, to the formation of aldosterone only by the glomerulosa. These findings imply the localization within specific areas of the cortex of some enzymes recently isolated from cortical tissue and shown to participate in corticoid biogenesis. They make it possible to outline an enzymic zonation of the adrenal cortex which would be characterized by the presence of both specific and common enzymic activities within the glomerulosa and the fasciculata-reticularis.

Thus, 17-hydroxylase activity would be found essentially in the cells of the fasciculata-reticularis, since progesterone increases the production of hydrocortisone in the presence of slices formed of this type of cells, but does not promote the formation of this steroid on incubation with glomerulosa cells. Conversely, 18-oxygenation would occur only in the cells of of the glomerulosa as indicated by the observation that progesterone, desoxycorticosterone, and corticosterone enhance the production of aldosterone by these cells but do not lead to its formation by fasciculata-reticularis cells. Finally, 3β-dehydrogenase activity and both 11- and 21-hydroxylase activities would be found throughout the cortex since 21-hydroxypregnenolone leads to an increase of aldosterone and hydrocortisone production in the glomerulosa and the fasciculata-reticularis respectively, and since the conversion of progesterone to hydrocortisone and aldosterone requires both 21- and 11-hydroxylations.

In recent years studies on the biogenesis of aldosterone have led to a number of conflicting data. In beef adrenal homogenates incubated with added glucose, fumarate, nicotinamide and ATP, Wettstein *et al.* have shown that the yield of aldosterone is increased by desoxycorticosterone but is unaffected or even decreased by progesterone and corticosterone. The authors concluded that their results indicated the occurrence of a sequence of hydroxylation in 21, 18, and 11 during the biogenesis of aldosterone. Kahnt reinforced this conclusion by demonstrating the formation of C^{14}-aldosterone upon incubation of C^{14}-desoxycorticosterone (23, 24).

At variance with these results are those of Rosemberg *et al.* (25) who showed that after perfusion of isolated calf adrenal with progesterone, desoxycorticosterone, and corticosterone, and subsequent biological measurement of the aldosterone fraction partially purified from the effluent, only progesterone resulted in a significant increase of the sodium-retaining activity. Corticosterone gave equivocal results and desoxycorticosterone was without action. These conclusions might receive some support from preliminary observations of small yields of C^{14}-aldosterone after perfusion of beef adrenal with C^{14}-progesterone (26). On the other hand, although Travis and Farrell showed that incubation of progesterone-4-C^{14} with beef

adrenal capsule strippings resulted in the production of aldosterone-4-C^{14} of approximately the same molar activity, incubation with corticosterone-4-C^{14} yielded aldosterone possessing a molar specific activity of up to 54% of corticosterone-4-C^{14} (27).

In the present study, incubation of beef glomerulosa tissue in the presence of identical amounts of progesterone, desoxycorticosterone and corticosterone have been observed to increase the formation of aldosterone to the same extent. The conversion factor of these three steroids to aldosterone is in the order of 5 to 7% in 2 hours of incubation irrespective of the three dose levels tested. Thus, the presence of an hydroxyl group in 21, as in desoxycorticosterone, in 21 and 11 as in corticosterone, or their absence from 21 and 11 as in progesterone, was not reflected in the amount of aldosterone formed. This observation suggests that oxygenation in the 18 position might be the limiting factor in the biogenesis of aldosterone.

The present results find strong support in the work of Ayres who demonstrated a comparable conversion of C^{14}-progesterone, C^{14}-desoxycorticosterone and C^{14}-corticosterone to C^{14}-aldosterone upon incubation of these steroids with strippings of ox adrenal cortex (28), and concluded that they might represent a major, though not necessarily the only pathway of aldosterone biosynthesis. The data presented in this paper agree fully with this conclusion. As a whole, they also support the concept of biogenesis formulated by the Worcester Group (29) since they do not exclude the existence of a common precursor of mineralo and glucocorticoids, but only emphasize that the nature of the end product of its metabolic transformation is fully dependent on the existence of its 18-oxygenation solely in the glomerulosa or of its 17-hydroxylation solely in the fasciculata-reticularis.

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FUNCTIONAL ZONATION OF THE ADRENAL CORTEX: SITE OF ACTH ACTION

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ABSTRACT

When ACTH or corticotropin-peptides were added to the incubation media of beef adrenal slices composed chiefly of glomerulosa tissue they did not significantly affect the total production of corticosteroids nor that of aldosterone. The same corticotropin preparations markedly increased the production of total corticosteroids and of hydrocortisone in the incubation media of slices composed of fasciculata-reticularis cells.

In the presence of fasciculata-reticularis slices, ACTH promoted the conversion of cholesterol to hydrocortisone. In the presence of glomerulosa slices, ACTH neither promoted the conversion of cholesterol to aldosterone nor did it increase the conversion of 21-hydroxypregnenolone and progesterone to aldosterone.

NUMEROUS attempts have been made to localize the site of action of ACTH within the different cellular zones of the adrenal cortex. These investigations, which in majority were based on the observation of histological changes of the cortex following ACTH stimulation, have led some to believe that ACTH controls principally the growth and the function of the fasciculata-reticularis cells (1, 2). But relying on similar histological criteria, a number of investigators have emphasized that the zona glomerulosa is far from being independent of pituitary and ACTH influence (3–6).

By a different approach, Sonenberg (7) has obtained evidence that after the administration of I^{131} labelled ACTH preparations to rats there was a definite preference for the radioactivity to concentrate at the level of the two inner cellular zones of the cortex.

The relative ease with which the zona glomerulosa of beef adrenal can be separated from the fasciculata-reticularis (8) made it possible to reinvestigate this problem by a more direct approach. Consequently, it was decided to study the production of corticosteroids by the glomerulosa and the fasciculata-reticularis of beef adrenal incubated in the presence of ACTH.

METHODS

The methods used in the following study are essentially the same as those described in detail in the preceding paper (8). However, in order to obtain sufficient amounts of

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steroid material without increasing the time involved in the processing of a greater number of slices, the period of incubation was extended to four hours. In each experiment the different corticotropins were weighed and dissolved in Krebs-Ringer bicarbonate medium at a concentration of 2 I.U. per cc. After the usual half-hour of preincubation, the slices were transferred to a set of clean beakers. The required amounts of corticotropin in solution were poured onto the slices which were gently mixed to insure a uniform absorption of the hormone. The incubation media were then adjusted to the proper volumes (10 cc per gm. of tissue incubated) with Krebs-Ringer bicarbonate solution. In the experiments where both ACTH and corticosteroid precursors were used, these steroids were first redissolved in small amounts of the incubation media to which the slices were then transferred. The ACTH was added and the media adjusted to their proper volumes.

RESULTS

In a single experiment, glomerulosa, fasciculata and reticularis slices were incubated separately in order to study the responsiveness of the zona reticularis to ACTH stimulation. The quantitative estimations of Δ^4-3

TABLE 1. EFFECT OF ACTH ON THE PRODUCTION OF STEROID MATERIAL BY THE DIFFERENT CELLULAR ZONES OF BEEF ADRENAL CORTEX

	Glomerulosa				Fasciculata				Reticularis			
	μg./100 gm. of tissue/hr.				μg./100 gm. of tissue/hr.				μg./100 gm. of tissue/hr.			
	U.V.[1]	BT.[2]	P.S.[3]	M.A.[4]	U.V.	BT.	P.S.	M.A.	U.V.	BT.	P.S.	M.A.
Controls	1030	750	20	130	450	375	270	4	400	270	180	0
ACTH	1290	880	50	115	1800	1560	1070	1	1020	740	490	0
% Increase over Control	25	17	150		300	310	295		155	170	172	

[1] Maximal absorption at 2400 Å.
[2] Blue Tetrazolium reaction.
[3] Porter-Silber reaction.
[4] Mineralocorticoid activity in the Simpson-Tait's assay expressed in μg. of aldosterone.

ketosteroids, α-ketolic steroids, 17-hydroxycorticosteroids, (Porter Silber chromogens) and sodium-retaining activity in the crude lipid extracts of their respective incubation media are presented in Table 1.

These data already suggest that the main site of action of ACTH on corticoid production is to be found in both the fasciculata and the reticularis zones, whereas ACTH has a very weak or almost no action on the production of steroids by the isolated zona glomerulosa. Thus, in the presence of ACTH, the formation of Δ^4-3 ketosteroids, α-ketolic and 17-hydroxysteroids by the fasciculata and the reticularis was increased by about 300% and 160% above that of the respective controls.

In the glomerulosa, the production of Porter Silber reacting material only was definitely enhanced by ACTH (150% above control value). But one should consider that with or without added ACTH this material was recovered from the incubation media of glomerulosa slices to the extent of only 3–4% of the total amount found in the incubation media of both fasciculata and reticularis slices. It is therefore probable that the signifi-

cant increase of Porter Silber reacting compounds induced by ACTH in the incubation media of the glomerulosa slices merely reflects the action of ACTH on the production of 17-hydroxysteroids by fasciculata cells incompletely separated from the glomerulosa slices.

Finally, ACTH did not affect the production of sodium-retaining material which, as previously shown, was found exclusively in the incubation media of glomerulosa slices.

CHROMATOGRAPHIC STUDIES

A series of studies was then undertaken to investigate the action of ACTH on the production of aldosterone and corticosterone by the zona glomerulosa and on the production of hydrocortisone and corticosterone by the fasciculata-reticularis. In these experiments, ACTH was added to the incubation media of both parts of the cortex at the dose level of 1.0 I.U. per gm. of tissue incubated for four hours. The results are presented in Table 2. They show that under the conditions of these experiments, the production of aldosterone by the zona glomerulosa was equal to 71 ± 16.0 (S.D.) μg./100 gm. of tissue/hour, and to 84 ± 19.5 after the addition of ACTH ($P > .1$). The production of aldosterone was not significantly affected by ACTH. Conversely, in the presence of ACTH, the amounts of

TABLE 2. ACTION OF ACTH[1] ON THE PRODUCTION OF ALDOSTERONE AND CORTICOSTERONE BY THE GLOMERULOSA AND ON THAT OF HYDROCORTISONE AND CORTICOSTERONE BY THE FASCICULATA-RETICULARIS OF BEEF ADRENAL

Steroid measured		Controls	ACTH[1]
		μg. of steroid/100 gm. of tissue/hour	
Glomerulosa	Aldosterone	83, 65, 55, 57, 98, 72: 71 ± 16.0[2]	101, 64, 63, 105, 98, 72: 84 ± 19.5
	Corticosterone	96, 109, 109, 21: 84	153, 112, 142, 62: 117
Fasciculata-reticularis	Corticosterone	14, 18, 18: 16	28, 43, 36: 35
	Hydrocortisone	55, 58, 38, 41, 40, 52: 48 ± 8.6	106, 189, 114, 117, 187, 245: 160 ± 55.0

[1] ACTH Nordic-Biochemicals: 1 I.U./gm. of tissue incubated 4 hours. ACTH increases significantly ($P < .001$) the production of hydrocortisone, but has no action on the production of aldosterone ($P > .1$).

[2] Standard Deviation.

hydrocortisone produced by the fasciculata-reticularis (160 ± 55.0 μg./100 gm. of tissue/hour) were very significantly increased above the control values (48 ± 8.6 μg.; $P < .001$).

In these experiments, the glomerulosa incubated in the absence of ACTH was observed to produce more corticosterone (mean of four values: 84 μg./gm. of tissue/hour) than the fasciculata-reticularis (mean of four values: 16 μg./gm. of tissue/hour). ACTH increased the production of this hormone by only 27% in the incubation media of the glomerulosa slices and by 112% in those of the fasciculata reticularis. The validity of these results regarding the lack of action of ACTH on the production of corticosterone by the glomerulosa is limited by the observation of great variations in the production of this hormone by both parts of the cortex upon extension of the incubation period (see discussion).

Farrell has reported that some corticotropin-peptides prepared by Bell increase the production of aldosterone in the adrenal vein blood of decapitated dogs (9).

Table 3 and 3a present the results of a series of incubations in which $\gamma_1+\alpha_2$, $\alpha_1+\alpha_2$ and δ_1, corticotropins were added to the incubation media of glomerulosa and fasciculata slices at dose levels varying between 0.01 and 12.8 I.U./gm. of tissue incubated/four hours. The total Δ^4-3 ketosteroids were measured in the crude lipid extracts of the incubation media in the three experiments; the total α-ketolic steroids only in two; aldosterone and hydrocortisone were estimated after paper chromatography. The re-

TABLE 3. ACTION OF CORTICOTROPIN-PEPTIDES[1] ON THE PRODUCTION OF CORTICOSTEROIDS BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL

Corticotropin (I.U./gm. of tissue)		Fasciculata-Reticularis (μg./100 gm. of tissue/hr.)			Glomerulosa (μg./100 gm. of tissue/hr.)		
		Crude lipid extract		Hydrocortisone	Crude lipid extract		Aldosterone[2]
		Δ^4-3 ketosteroids[2]	BT. Reacting compounds		Δ^4-3 ketosteroids[2]	BT. reacting compounds	
Control		320	400	40	1000	640	99
$\gamma_1+\gamma_2$	.2	600	630	108	1200	1100	103
	.8	920	800	150	1200	1000	133
	3.2	1200	1070	216	1330	1000	150
	12.8	1560	1160	363	1600	1400	130
Control		373	431	47	720	530	83
δ_1	.2	1030	890	195	940	540	83
	.8	1200	980	253	920	690	89
	3.2	1510	1170	345	1140	1025	123
	12.8	1860	1350	387	1200	985	130

[1] Corticotropin-Peptides Lederle. Fraction $\gamma_1+\gamma_2$: Lot No. 1079-1. Fraction δ_1: Lot No. 1079-24.

[2] Measured at 2400 Å.

TABLE 3a. ACTION OF CORTICOTROPIN-PEPTIDES[1] ON THE PRODUCTION OF CORTICOSTEROIDS BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL

Corticotropin I.U./gm. of tissue	Fasciculata-Reticularis (μg./100 gm. of tissue/hr.)		Glomerulosa (μg./100 gm. of tissue/hr.)	
	Δ^4-3 keto-steroids[2]	Hydrocor-tisone	Δ^4-3 keto-steroids[2]	Aldosterone
0.01	210	90	440	90
0.1	450	190	520	100
1.0	890	250	670	100
10.0	1100	370	720	95
Control	140	60	380	91

[1] Corticotropin-Peptides Lederle: Fraction $\alpha_1 + \alpha_2$.
[2] Measured at 2400 Å.

sults show that only in the incubation media of fasciculata-reticularis did these three ACTH peptides markedly increase the production of Δ^4-3 ketosteroids and α-ketolic steroids, and that the degree of the response was proportional to the dose of corticotropins added. The production of hydrocortisone increased from control values of 40, 47 and 60, to 363, 387 and 370 μg./100 gm. of tissue/hour in the presence of the greatest dose of each corticotropin. As opposed to these results, the production of total Δ^4-3 ketosteroids and of α-ketolic steroids, as well as that of aldosterone by the glomerulosa slices was little affected by any one of the three ACTH peptides over the range of dose levels used, and in no case could a dose-response relation be established.

Since the work of Stone and Hechter on perfusion of beef adrenals, it has been generally recognized that one of the main actions of ACTH on corticosteroid biogenesis may lie in its control of an enzymic sequence essential for the conversion of cholesterol to Δ-5 pregnenolone and, consequently, for an increased formation of hydrocortisone and corticosterone (10). It was therefore considered of interest to study whether ACTH would promote the formation of aldosterone from cholesterol in the zona glomerulosa, or would promote the conversion of cholesterol to other corticosteroids in the fasciculata-reticularis exclusively. Hence, glomerulosa and fasciculata-reticularis slices were incubated in the presence of added cholesterol, 21-OH pregnenolone or progesterone, both with and without ACTH. The results of these experiments are reported in Table 4. They show that the production of aldosterone was unaffected by addition of cholesterol but was increased in the presence of 21-OH pregnenolone and progesterone. The addition of ACTH did not affect the conversion of 21-OH pregnenolone or progesterone to aldosterone, nor did it promote an increase of aldosterone in the presence of cholesterol. The production of hydrocortisone by the fasciculata-reticularis was increased above the control values by 170% in the presence of ACTH alone, and by 320 to 490%

TABLE 4. PRODUCTION OF ALDOSTERONE BY THE GLOMERULOSA AND OF HYDROCORTISONE BY THE FASCICULATA-RETICULARIS OF BEEF ADRENAL INCUBATED WITH PRECURSORS IN BOTH THE PRESENCE AND THE ABSENCE OF ACTH

		Controls	ACTH[1]	Cholesterol[2]		21-OH Pregnenolone[2]		Progesterone[2]	
				Alone	+ACTH	Alone	+ACTH	Alone	+ACTH
μg./100 gm. of tissue/hour.	Aldosterone	88 ± 11[3]	99 ± 16	115	113	310	240	330	320
				102	115	299		320	294
	Hydrocortisone	51 ± 7	136 ± 38	81	240	117	210	520	483
				62	340	144		600	742

[1] Nordic Biochemicals: 1 I.U./100 mg. of tissue incubated 4 hours.
[2] Added at the dose level of 10 μg./100 mg. of tissue incubated 4 hours.
[3] Standard deviation of the mean.

in the presence of both ACTH and cholesterol. Finally, the increased production of hydrocortisone promoted by progesterone was not affected by the addition of ACTH.

DISCUSSION

As early as 1940 Swann (11) expressed the view that hormones controlling mineral metabolism could be produced by the zona glomerulosa since the histological aspect of this zone as well as mineral metabolism are little affected by hypophysectomy. Conversely, the two inner zones of the cortex could be the site of production of hormones controlling carbohydrate metabolism since the profound disturbance of this metabolism after hypophysectomy is accompanied by marked atrophy of these zones. This view was fully supported by the data of Bergner and Deane (1) who showed that the histochemical changes observed in the adrenal cortex of ACTH-treated rats were mostly located within the fasciculata-reticularis. More recent works have demonstrated that prolonged treatment of the dog with glucocorticoids is followed by a marked decrease in the rate of production of hydrocortisone in the adrenal vein blood and accompanied by atrophic changes in the fasciculata-reticularis without detectable alteration of the zona glomerulosa (2). Since it is known that after cortisone administration the synthesis of pituitary ACTH is almost fully depressed (12) the over-all impression gained from this more direct approach is that in the dog

also the site of action of ACTH is most likely limited to the cells of the fasciculata-reticularis.

The independence of the zona glomerulosa from pituitary control has been challenged by a number of histological studies. Thus Selye and Stone noted even in short term experiments in rats that a large dose of ACTH can stimulate growth of the zona glomerulosa (3). In hypophysectomized rats, Lever has observed a regression of all three cortical zones and conversely marked changes in the histological aspect of the zona glomerulosa after ACTH administration (4). Complete or almost complete depletion of sudanophilic elements of the zona glomerulosa was also observed by Wexler in rats treated with certain ACTH preparations (5). Following extensive histological studies in mice, Chester Jones expressed the view that the zona glomerulosa has probably no specific function in the elaboration and secretion of mineralocorticoids (6). From these data alone it appears difficult to draw definite conclusions on the site of action of ACTH on the adrenal cortex.

Comparing the production of aldosterone by the zona glomerulosa and that of hydrocortisone by the fasciculata-reticularis, the present work strongly suggests that the action of ACTH on corticosteroid biogenesis is mostly demonstrable in the presence of cortical slices made up entirely of fasciculata-reticularis cells. This conclusion is based on observations that three of the well-recognized effects of ACTH on corticoid production are clearly and exclusively demonstrable in the presence of fasciculata-reticularis slices: 1) in the presence of ACTH the production of hydrocortisone by the fasciculata-reticularis was markedly enhanced; 2) a linear response relationship between the dose of ACTH used and the increase of hydrocortisone production was demonstrable; 3) ACTH promoted the increase of hydrocortisone formation in the presence of added cholesterol.

The possible action of ACTH on the biogenesis and secretion of aldosterone has fostered a number of investigations. Singer has demonstrated that after hypophysectomy the secretion of aldosterone in the adrenal vein blood of rats is decreased to 25% of normal, and that the secretion of this hormone is returned to normal by treatment with ACTH (13). In hypophysectomized dogs, secretion of aldosterone in the adrenal vein blood effluent was observed by Farrell to be reduced by 1/3 of the normal value (15). The same author was the first to provide evidence that the rate of aldosterone secretion is depressed to about 30% in decerebrated dogs (16) and brought back to normal or above normal values after intravenous perfusion of crude beef diencephalon extracts (17). A less marked but comparable effect on hydrocortisone secretion was recorded. In the same animal preparation a linear response could be obtained between doses of δ_1 and β corticotropin-peptides and the amounts of aldosterone secreted in the adrenal vein blood (9). Perfusion of beef adrenals (18) or incubation of rat

(19) as well as human (20) adrenals with ACTH has led to some increase in the production of aldosterone although the results obtained with rat adrenals have often been found to vary with the preparation of ACTH used (19). Finally, the production of aldosterone by beef adrenal slices was increased by certain posterior pituitary extracts which had only a questionable action on the production of hydrocortisone; in these experiments ACTH had the opposite effect (21). These apparently conflicting results have led to the hypothesis, first expressed by Farrell (9), that a humoral factor different from ACTH might be responsible for the regulation of aldosterone secretion. The existence of such a factor, its presence in widely varying amounts as a contaminant of many pituitary hormones and (possibly) hypothalamic extracts, and the degree to which its action on aldosterone secretion might be interfered with by the action of ACTH on glucocorticoid secretion, could explain the apparent contradiction of results obtained in studies of the action of ACTH on aldosterone secretion.

The results of the present investigation show: 1) that none of the corticotropic preparations studied had significant action on the production of aldosterone by the isolated zona glomerulosa, 2) that the production of this hormone is unaffected by addition of cholesterol alone or by both cholesterol and ACTH, and finally, 3) that ACTH has no demonstrable action on the conversion of possible precursors of aldosterone such as pregnenolone and progesterone.

In these experiments corticotropin-peptides were not able to increase the production of aldosterone by more than 50% of the control values. Such an increase is felt to be too close to the possible range of variation in the production of aldosterone by the controls to be of significance. The lack of action of $\delta - 1$ corticotropin on the production of aldosterone may seem surprising since *in vivo* the same preparation was demonstrated by Farrell to increase markedly the secretion of this hormone in the adrenal vein effluent of decapitated dogs, and that in both his and the present work the quantities used lay in the same range. It is difficult to explain this result solely by a lack of sensitivity of the *in vitro* method, since previous work has demonstrated a two to threefold increase of aldosterone secretion by whole slices of beef adrenal cortex incubated in the presence of posterior pituitary preparations (21).

To postulate a loss of potency of δ_1 corticotropin which was kept in a solid state at 5° C for a few weeks would provide an explanation which we do not think likely. In a preliminary experiment, undertaken with the aim of assessing crudely the possible differences of potency of these 3 corticotropin-peptides on the production of aldosterone and hydrocortisone, an incubation was performed using each preparation at one single dose level (2 I.U./gm. of tissue). In this experiment, which is not reported quantitatively here, the production of aldosterone was increased roughly 100%

above the control values by the three corticotropins used. These results could not be reproduced when each of these corticotropins was tested in the experiment reported here, despite the dose range used (0.01–12.8 I.U./gm. of tissue).

The data presented strongly support the concept of functional zonation of the adrenal cortex, as first established by Deane, by offering direct proof of the main action of ACTH on the two inner zones of the adrenal cortex. They cannot be interpreted as supporting or disproving the existence of a humoral factor controlling the secretion of aldosterone by the zona glomerulosa, the existence of which, as a possible contaminant of pituitary preparations, is suggested by *in vivo* studies (9).

The final proof that the secretion of corticosteroids by the zona glomerulosa is totally independent of ACTH might rest with studies on the production of corticosterone by this zone of the cortex. Preliminary data suggest that ACTH might have an action both on the production of corticosterone and on the conversion of cholesterol to corticosterone throughout the adrenal cortex though, possibly, to a greater extent in the fasciculata-reticularis.

However, in the present study it has often been difficult to measure corticosterone after incubation of the slices for four hours with or without added ACTH. It was repeatedly noticed that at the end of 2 hours of incubation the amounts of corticosterone recovered from the incubation media were approximately equal in both parts of the cortex. However, by increasing the time of incubation to 4 hours or more, corticosterone progressively disappeared from the media. This was observed whether in the absence of added precursors or in the presence of progesterone. In one incubation with this steroid, the amounts of corticosterone produced by the glomerulosa and the fasciculata-reticularis were respectively equal to 280 and 1560 μg./100 gm. of tissue after 4 hours of incubation, whereas no corticosterone was recovered from the incubation media of glomerulosa, and only 60 μg./100 gm. of tissue from that of the fasciculata-reticularis after 8 hours of incubation.

Perfusions of beef adrenal have shown that ACTH increases the production of corticosterone (22). On the other hand, the present data indicate both the importance of corticosterone as a possible precursor of aldosterone in the zona glomerulosa (8), and the insignificant action of ACTH on aldosterone biogenesis. The existing data admit at least two different possibilities: either that both aldosterone and the fraction of corticosterone produced by the glomerulosa belong to a pathway of steroid biogenesis which by-passes cholesterol and is slightly or not at all affected by ACTH; or that throughout the cortex, corticosterone is under ACTH control—which could imply that this steroid is not an obligatory pathway in the biogenesis of aldosterone.

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METABOLISM OF THYROXINE DURING PROLONGED KIDNEY CORTEX INCUBATION[1]

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ABSTRACT

A study has been carried out of the metabolic changes in thyroxine, as the hormone maintained the oxygen consumption of rat kidney cortex slices incubated for three days at 5°C. Tetraiodothyroacetic acid (Tetrac) and iodide were rapidly produced, with triiodothyroacetic acid (Triac) and small amounts of a thyroxine conjugate appearing later. When Tetrac was added instead of thyroxine, conjugation was more rapid, but no Triac was formed. Addition of Triiodothyronine (Trit) resulted in Triac formation, as well as maximal production of both glucuronic and sulfuric acid conjugates. No Trit was encountered, even with careful bidimensional chromatography; thus, renal deamination and decarboxylation of Trit must dispose of it as fast as it is produced from thyroxine. No correlation could be obtained between production of any metabolite of thyroxine and its effect on kidney cortex metabolic rate. These results suggest that there is a response of some labile factor in the cell rather than production of an "active" thyroxine derivative.

IT HAS previously been shown (1) that, during prolonged incubation of rat kidney cortex at 5° C, added thyroxine had the striking effect of maintaining oxygen consumption of the tissue for as long as 72 hours. This action was especially marked in the presence of alanine or proline, and was completely eliminated by the addition of tryptophan. The present study is concerned with the metabolism of the thyroxine. Since there is considerable current interest in the possible production of "active" forms of the hormone, 3,5,3′-triiodothyronine (Trit) and 3,5,3′,5′-tetraiodothyroacetic acid (Tetrac) were also studied.

METHODS

The experimental conditions were the same as those reported by Lindsay and Barker (2). Slices of kidney cortex from thyroidectomized rats were placed in Warburg vessels in Ringer-alanine-glucose or Ringer-proline-glucose and held at 5° C, oxygen consumption being measured at 37° C before incubation (Day 0) and after 1, 2 and 3 days. To the 10 μg. thyroxine present per ml. of medium was added radioiodine-tagged thyroxine (Abbott), about 100,000–250,000 cpm per flask. Comparative determinations showed no effect of this much radioactivity on the oxygen consumption. Variations in this gen-

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eral procedure included terminating the incubation at earlier times, incubation for various lengths of time before addition of thyroxine, or substitution of a thyroxine-free solution. In addition, radioiodine-tagged Trit or Tetrac together with carrier in amounts equimolar to 10 μg. thyroxine/ml. were substituted for the thyroxine.[2]

Immediately following the desired incubation, the contents of each set of duplicate vessels were pooled and centrifuged at 0° C. The clear supernatant medium was poured off and kept cold for counting and chromatography. The tissue pellet was washed three times with cold Ringer solution, being separated each time by centrifugation at 0° C. Each sample was then repeatedly extracted with small portions of methanol-1% ammonium hydroxide in a small Potter-Elvehjem glass homogenizer kept in ice. The pooled extracts were filtered and used either directly, or after concentration at 40° C under reduced pressure. Although the latter procedure has been found not to alter the distribution of iodinated compounds, we have preferred to start with sufficient radioactivity to avoid this extra step of handling. It was necessary to use the incubation medium without concentration to avoid adsorption of organic iodine on the large quantity of solid. We have already reported that electrolytic desalting results in deiodination of thyroxine and related compounds (3).

Before chromatography, aliquots of the tissue extracts and incubation media were evaporated and counted dry with a thin end-window Geiger-Müller tube. Suitable quantities were then chosen for treatment by ascending chromatography with the following solvent mixtures: butanol-water-acetic acid (78:17:5), butanol-water-ammonium hydroxide (250:178:72), tertiary amyl alcohol-water-ammonium hydroxide (50:40:10), or collidine-water (100:35) in an atmosphere of ammonia from concentrated ammonium hydroxide. Quantitative Geiger-Müller scanning of the activity in the developed but unstained chromatogram was carried out along a marked grid. Autoradiograms were obtained on Kodak No-Screen x-ray film. Chemical demonstration of spots was carried out with sprays of ninhydrin for amino acids or diazotized sulfanilic acid for phenols (4). Iodine-containing compounds were revealed with a ceric sulfate-arsenious acid stain (5). After qualitative identification, each substance present was expressed quantitatively in terms of percentage of the total radioactivity added as thyroxine, Tetrac, or Trit.

RESULTS

Metabolism of thyroxine. The three-day maintenance of kidney cortex metabolic rate in the presence of thyroxine is demonstrated in Figure 1A for the composite of several experiments involving incubation in Ringer-alanine-glucose. The control vessels (B.) showed good respiration for the first day, but fell off rapidly after that time. There was a striking contrast when thyroxine was present in the incubation medium throughout the entire three-day period.

The distribution of radioactivity in the tissues and incubation solutions of these experiments is shown in Figure 2 for various lengths of time. The hormone was rapidly taken up by the tissue, 32% being thus located after only 20 minutes at 37°, and 65% after 1 hour. Metabolic alterations of the thyroxine were also rapidly occurring, with a small amount of Tetrac

[2] Generous quantities of these substances were provided by Dr. R. Pitt-Rivers of the National Institute for Medical Research, Drs. R. I. Meltzer and R. L. Kroc of the Warner-Lambert Research Institute, Dr. A. E. Heming of the Smith Kline and French Laboratories, and Dr. H. L. Fevold of the Baxter Laboratories.

appearing in both tissue and solution after 20 minutes, as well as 5% of inorganic iodide. These actions continued during the rest of the first hour.

By the end of one day of incubation (2 hours at 37° C and 23 hours at 5°), the total tissue radioactivity had begun to fall. The distribution of activity was essentially unchanged, but the amount now present as acetic acid derivatives ("Ac") had increased, especially in the tissue. At this time, Triac first clearly appeared in the tissue. As the incubation was extended through four days, the amount of these acetic acid derivatives increased, as well as the proportion present as Triac. Tissue activity was evidently responsible for the necessary deamination and decarboxylation, since there was always more acetate derivative in the tissue than in the solution. Care-

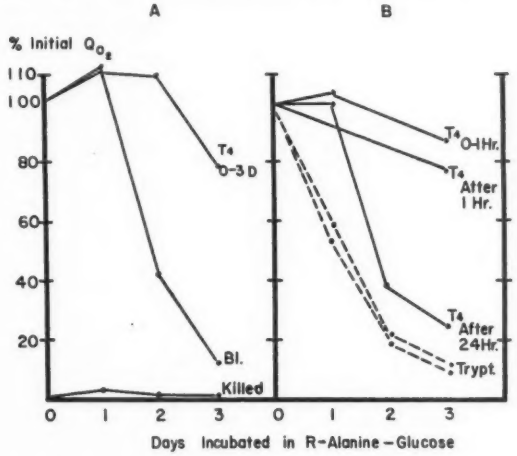

FIG. 1. Oxygen consumption of rat kidney cortex slices in Ringer-alanine-glucose solution. Measurements made at 37° C on days 0, 1, 2, 3; incubation otherwise at 5° C. "T_4" indicates solution containing 10 μg. L-thyroxine/ml.; "Bl" control solution. "Trypt" solutions contained added tryptophan, thyroxine flasks the lower dotted line. See text for further explanations.

ful bidimensional chromatography consistently failed to reveal the presence of Trit at any time, from 20 minutes to three days (Fig. 3). Since there was always more Tetrac than Triac, we originally concluded that the former was the source of the latter, as well as of the activity found as iodide. However, it will be shown later that added Tetrac yielded no Triac, whereas added Trit did.

In some experiments, it was possible to see two thin lines of radioactivity on the autoradiograms between the origin and the iodide. As the scanning was ordinarily done on acid-developed chromatograms of incubation solutions, these compounds were included in the thyroxine fraction. More exact counting indicated that these amounted to 4% of the total activity at most.

By analogy with other results to be presented, (see Fig. 8) it is believed that these are conjugates of thyroxine.

Experimental changes in the exposure of kidney slices to thyroxine were introduced, as shown in Figure 1B. It can be seen that, when thyroxine was present in the solution only for the first hour of incubation (at 37° C) or

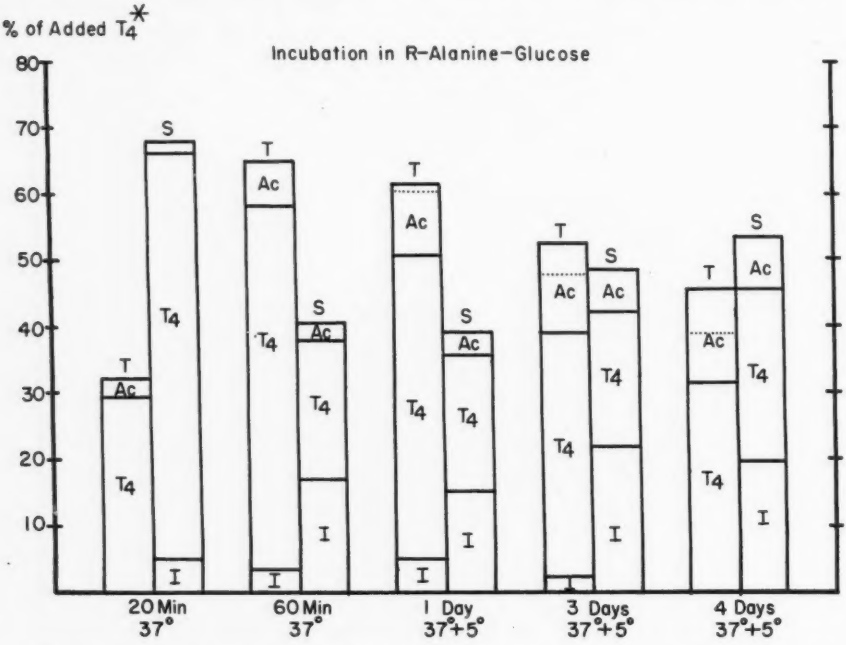

FIG. 2. Metabolism of added radiothyroxine under certain of the conditions shown in Fig. 1 The first two columns show the redistribution of radioactivity between solution (S) and tissue (T) after 20 and 60 minutes at 37° C, the remainder at various times of longer incubation at 5° C combined with respiration measurements at 37° C. Fractions are indicated as follows:

T_4, thyroxine; Ac, Tetrac plus Triac (latter above dotted line when present); I, inorganic iodide.

when the hormone was not added until after that hour, the 3-day maintenance of metabolism was nearly as great as when thyroxine was present throughout the entire time. In contrast, withholding thyroxine until after the first 24 hours of incubation gave results no different from the control solution. When tryptophan was added, the thyroxine action was completely blocked. The tissue subjected to 100° C for 10 minutes did not consume significant amounts of oxygen at any time.

Figure 4 shows that tryptophan did not alter the metabolism of thyroxine. Even delaying the addition of thyroxine until after one day produced the quantitative differences one would expect from the shorter time available

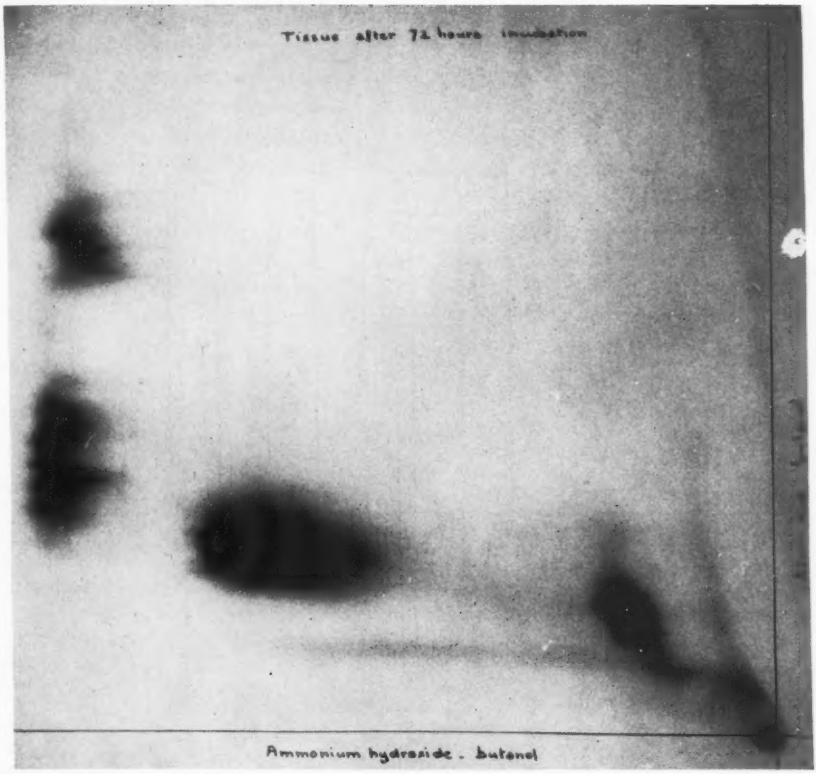

FIG. 3. Autoradiogram of bidimensional chromatogram of extract of kidney tissue slices incubated with radiothyroxine for 72 hours. Origin lower right, solvents as shown. References identified spots present as:

Triac
Tetrac Thyroxine
Iodide
Origin

for tissue action on the hormone, namely, a greater proportion of thyroxine in the tissue, and lower levels of Tetrac and iodide. When thyroxine was present only for the first hour, the tissue retained 60% of the radioactivity, mostly as thyroxine itself (Fig. 5). During the succeeding 3 days, there was a great loss of this thyroxine into the new solution added without thyroxine. This lower tissue hormone level resulted in a smaller production of Tetrac. In all these instances, it is apparent that metabolic changes were produced in the thyroxine as an amino acid and that the production of Tetrac or Triac cannot be considered an "activation," nor does the presence of Trit seem to be necessary.

The results with the heat-denatured tissue were surprising (Fig. 4).

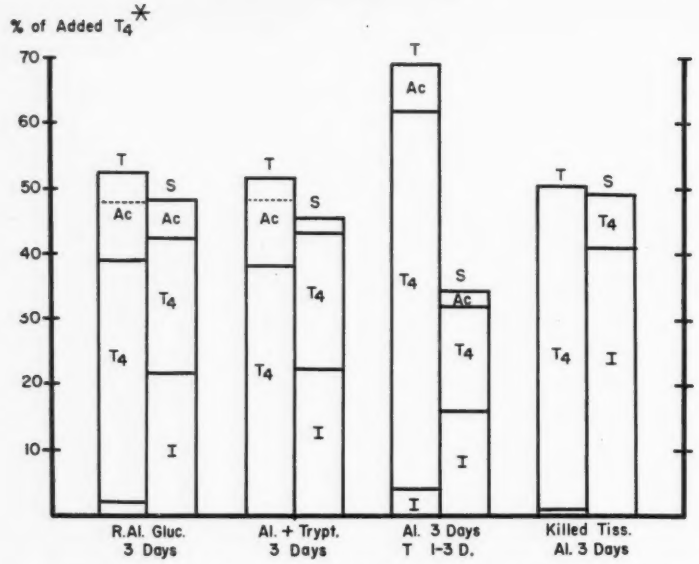

FIG. 4. Metabolism of radiothyroxine during kidney cortex incubation under certain of the conditions shown in Fig. 1. Symbols as in Fig. 2.

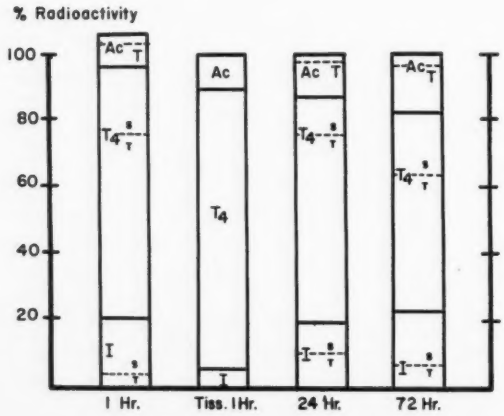

FIG. 5. Redistribution of the radioactivity present in kidney slices after 1 hour's exposure to radiothyroxine at 37° C during a subsequent 3 days' incubation in Ringer-alanine-glucose. Column 1: original distribution of activity in tissue (T) and solution (S) at the end of 1 hour at 37° C. Column 2: tissue portions of column 1 expanded to 100%. Columns 3 and 4: redistribution and metabolism of radioactive materials originally present in the tissue (column 2) after incubation at 5° C for 24 and 72 hours, respectively. Symbols as before: T_4, thyroxine; Ac, Tetrac plus Triac; I, inorganic iodide.

There was a rapid "uptake" of thyroxine, and even after 3 days, the washed killed tissue contained more than four times the outside concentration of thyroxine. Furthermore, twice as much iodide had been formed as with the viable tissue. Extended study of this phenomenon, to be reported elsewhere, has shown that thyronine is one principal product of deiodination.

When proline was the added amino acid, the metabolism of thyroxine was very nearly the same as in the presence of alanine. Some of these results are presented in Figure 6, for comparison with alanine. There was

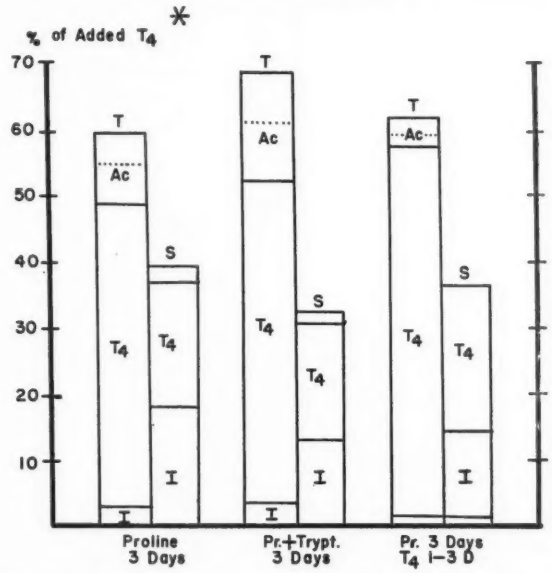

FIG. 6. Metabolism of added radiothyroxine (T_4*) by kidney cortex slices incubated in Ringer-proline-glucose (Columns 1, 3) or Ringer-proline-glucose with tryptophan (Column 2). In column 3, thyroxine was present only after the first 24 hours of incubation. Symbols designating partitioned radioactivity as in Fig. 2.

approximately the same amount of thyroxine present in the tissue at the end of three days in both, 43% in proline and 35% for alanine. Tryptophan blocked thyroxine maintenance, as before, but did not interfere with the production of Tetrac, Triac and iodide. Again, delaying the addition of thyroxine until after one day of incubation, completely eliminated its effect on kidney metabolism, but introduced no more than the quantitative decrease in thyroxine products expected from the shorter time.

Metabolism of Tetrac. Since Tetrac appeared as an early metabolic product of thyroxine, its effect was next studied. Figure 7A shows that Tetrac maintained oxygen consumption of incubated kidney cortex slices in much the same manner as thyroxine: 119% of the initial Q_{O_2} value after 24 hours, 101% after 48, and 78% after 72.

Chromatography of the tissue and medium after 72 hours of incubation (Fig. 7B) revealed most of the radioactivity in the former to be the original Tetrac, with a very small amount of iodide and another spot of low R_f in alkaline solvents. In addition to a smaller amount of Tetrac and a larger quantity of iodide, the medium contained enough of the low R_f compound to account for 10% of the total radioactivity (see Fig. 8). When the incubation solution was chromatographed using collidine-ammonium hydroxide, an R_f of 0.31 was obtained, strongly suggesting a glucuronic acid conjugate. A preparative chromatogram was therefore run, using butanol-

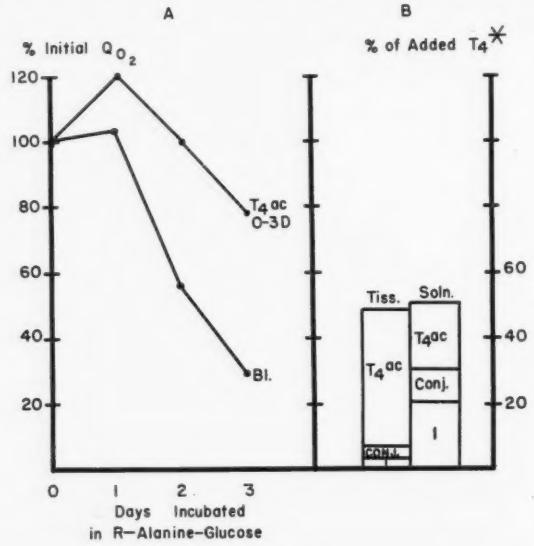

FIG. 7. Tetrac addition to kidney slices. A. Maintenance of oxygen consumption with added Tetrac (T_4 ac). B. Metabolism of radioactive Tetrac during 3 days' incubation. "Conj." indicates glucuronic acid conjugate of Tetrac.

ammonium hydroxide and the spot was eluted with methanol-ammonium hydroxide. After condensation *in vacuo* below 40° C, the material was taken up in acetate buffer at pH 5 and incubated 2 hours at 37° C with a liver β-glucuronidase (Warner-Chilcott Ketodase®). Under these conditions there was a small amount of deiodination, but chromatography in collidine-ammonia and butanol-ammonia showed disappearance of the glucuro-conjugate and appearance of Tetrac. It can thus be concluded that 12% of the Tetrac originally present was conjugated with glucuronic acid, most of this reappearing in the solution. The second conjugate which can be seen faintly in Figure 8 is believed to be a sulfate, although the amounts present were too small to permit isolation.

Repeated mono- and bidimensional chromatograms were run with in-

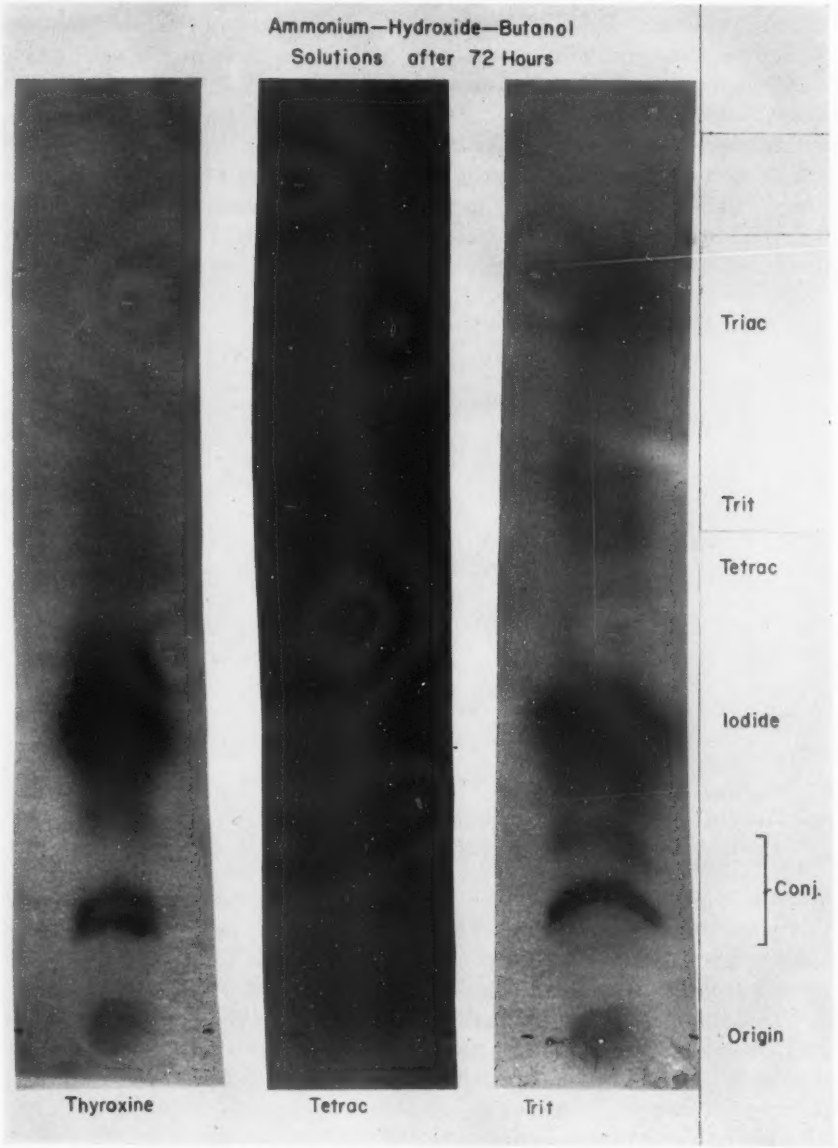

FIG. 8. Autoradiograms of three different solutions after 72 hours' incubation with rat kidney cortex slices, chromatographed in butanol-ammonium hydroxide. Exposure times were not consistent from one strip to another, but relative amounts of radioactivity in the conjugates and inorganic iodide are comparable. The spots are indicated upwards from the origin at the bottom: conjugates (Conj.), inorganic iodide, Tetrac in the thyroxine and Tetrac media, Trit and Triac with Trit medium.

cubation solution and tissue extracts. In no case was it possible to see a Triac spot even on bidimensional autoradiograms, interpreted as meaning that less than 2% of the Tetrac could have been partially deiodinated to Triac. The appreciable quantities of Triac formed in the thyroxine experiments, therefore, could not have come from Tetrac.

Just as with thyroxine, tryptophan blocked the Tetrac maintenance entirely, but did not alter either deiodination or glucuro-conjugation. Thus, this is additional evidence that the oxygen consumption effect of thyroactive materials is exerted independent of the metabolism of these.

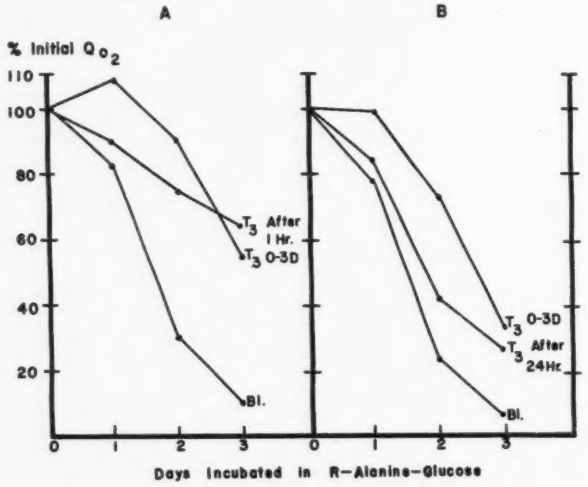

FIG. 9. Effect of Trit (T_3) on maintenance of oxygen consumption of kidney slices in Ringer-alanine-glucose. Other symbols as in Fig. 1.

Metabolism in the presence of 3,5,3'-Triiodothyronine (Trit). Although Trit was not actually encountered at any time during incubation of the kidney cortex slices with thyroxine, the formation of Triac from thyroxine but not from Tetrac made it imperative to evaluate Trit. Figure 9 shows that this product also was capable of producing oxygen consumption effects when it was added at the start of incubation or after the preliminary hour at 37° C. Maintenance, although clearly better than the blank, was not as effective as with thyroxine.

Trit is known to be more active than thyroxine *in vivo*, that is, the same metabolic stimulation can be obtained with one-quarter to one-tenth as much of the former. This does not occur *in vitro*, since the response to various concentrations of Trit is the same as with thyroxine. Trit has also been shown to be metabolized and eliminated from the body more rapidly than thyroxine (6). Figure 10 presents studies on the metabolism of Trit by kidney slices showing 60% penetration by 60 minutes, no more rapid than that of thyroxine. This value had fallen to 50% by 72 hours. Triac was

formed at about the same rate as Tetrac plus Triac in the case of thyroxine for the first 48 hours, although the 72-hour figure was greater with Trit. Iodide also appeared from both in approximately equal amounts. There was only 37% of free Trit remaining after 72 hours, in contrast to 57% of the thyroxine. The disparity between the amounts left in the tissues was not as great, being 30% for Trit and 36% for thyroxine. It is not certain

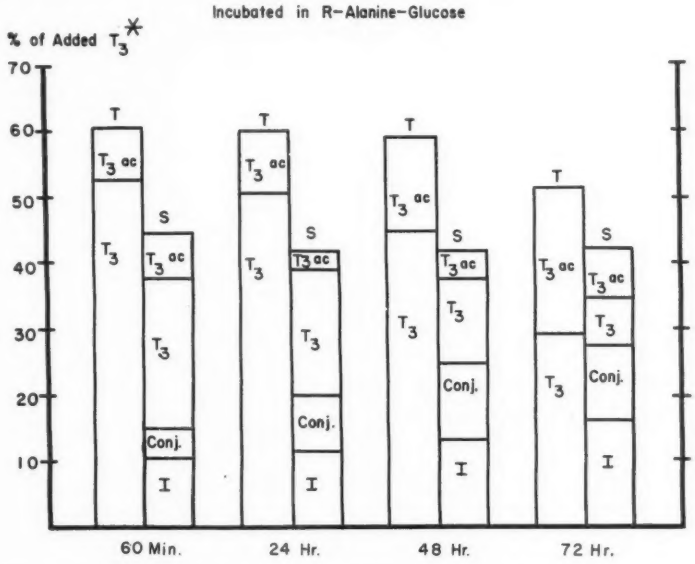

FIG. 10. Metabolism of radioactive Trit (T_3^*) after various times of incubation as in Fig. 8. Other symbols: T_3, Trit; T_3 ac, Triac; Conj., glucuronic and sulfuric acid conjugates of Trit; I, inorganic iodide.

that any of these differences is adequate to explain the poorer *in vitro* maintenance with Trit, or the greater metabolic effects obtained *in vivo*.

Chromatograms of the incubation solutions in butanol-ammonium hydroxide revealed two spots between the origin and iodide (Fig. 8). Run in collidine-ammonium hydroxide, treatment with bacterial β-glucuronidase (Sigma) eliminated the spot of R_f 0.3 and increased the amount of Trit. This material was, therefore, the glucuroconjugate of Trit. The second material, closer to iodide, appeared to be the sulfoconjugate of Trit, similar to that found in rat bile after administration of Trit to the animal (7). About 5% of the added Trit was conjugated by these two mechanisms during the first hour, increasing to 22% by the end of the experiment. Neither conjugate was found in the tissue itself, nor was iodide.

Autoradiograms of the 72-hour bidimensional chromatograms presented an unexpected picture. From the tissue extracts, besides the anticipated Trit and Triac, there was obtained a faint spot of R_f 0.84 in butanol-acetic acid and 0.84 in butanol-ammonium hydroxide. An acid metabolite of

Trit other than Triac of 3,3′-diiodothyroacetic is suspected. In the incubation solution, besides Triac, the conjugates mentioned, and iodide, there was a very faint spot of R_f 0.81 in butanol-acetic and 0.20 in butanol-ammonium hydroxide, for which no identification can be offered.

DISCUSSION

There was an increase of the different metabolites of thyroxine during the time incubation of rat kidney slices. Similar results were obtained with Trit. The acetic acid derivatives first accumulated in the tissue, then the excess passed into the incubation medium. Although Yamazaki and Slingerland (8) did not encounter any acetic acid derivatives, these have also been identified as resulting from homogenate action on thyroxine by Tomita *et al.* (9). Other components, such as the conjugates and iodide, passed rapidly out of the tissue and were found in much higher concentration in the solution.

It has been well established that the liver can conjugate thyroxine and related compounds (10). Taurog *et al.* (11) demonstrated that kidney was also capable of a smaller level of glucuro-conjugation of thyroxine. The present studies not only confirm this renal activity but show that Tetrac is more readily conjugated than thyroxine, and Trit the most extensively of the three. The autoradiograms strongly suggest that the last compound readily forms a sulfoconjugate as well as the glucuronic. The only obvious structural basis for these differences in extent of conjugation would be in the case of Trit, where removal of the 5′-iodine would give greater freedom of approach to the 4′-hydroxyl group.

Thyroxine maintenance of respiration in these experiments could be achieved if the hormone was added after the first hour at 37° C, but not after 24 hours at 5°. The metabolites produced from thyroxine were the same under all these circumstances. This strongly suggests that thyroxine had an important effect on something in the kidney cells which remained intact for about one hour at 37° C but was lost by 24 hours at the lower temperature, rather than involving some "active form" of the hormone (12). This concept is supported by two additional pieces of evidence. Added tryptophan completely blocked any thyroxine maintenance, but did not interfere with the production of Tetrac, Triac and iodide. Furthermore, addition of Tetrac instead of thyroxine did not give significantly different results. Other experiments, not reported here, have ruled out Triac as peculiarly active.

The source of the Triac encountered during incubation with thyroxine requires some consideration. Tetrac always appeared first, and always was present in greater amounts than Triac, leading one to suspect Tetrac as the immediate source. However, added Tetrac yielded no Triac, whereas Trit did, and in considerable quantitity, equal to Tetrac plus Triac from thyroxine. Although no trace of Trit was found during the thyroxine ex-

periments, it seems probable that it must have been formed and immediately deaminated and decarboxylated. There always remains the unprovable possibility that Tetrac as produced from thyroxine is more labile than exogenous Triac added to the incubation medium.

From many of the figures, it is evident that deiodination is a prominent feature of the metabolism of thyroxine, as well as of Tetrac and Trit, by kidney tissue. The most unusual characteristic was that heat-killed slices showed twice as much deiodination at the end of 3 days as did viable tissue. This point will be pursued more in detail elsewhere, but it can be noted that non-enzymic deiodination has been shown for liver and pancreas by Lissitzky *et al.* (13). Larson *et al.* (14) and Tata (15) reported thermolabile deiodination by kidney, and by brain and muscle, respectively. Under our conditions, the summated deiodination processes, if more than one, were less active in viable tissue.

Considerably more iodide appeared in every experiment than could be accounted for on the basis of triiodo-compounds formed. Presumably, complete deiodination of the respective parent compounds must have occurred, since the sensitive ceric-arsenious stain revealed no iodine spots corresponding to non-radioactive 3,5-iodinated compounds which might have been produced. Although thyronine has been identified as the principal product from heat-killed deiodination of thyroxine, no identification of Pauly-reactive compounds has been possible in the experiments on living tissue, because of the small quantities available, and the relative insensitivity of the color reaction as compared to the iodide test.

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THE EFFECT OF TOLBUTAMIDE ON THE DIABETOGENIC ACTION OF SOMATOTROPIN[1]

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ABSTRACT

The administration of tolbutamide to dogs by mouth prevents the diabetogenic response which ordinarily occurs with the daily intramuscular injection of somatotropin.

THE mechanism whereby the daily administration of somatotropin to the dog results in the development of a temporary diabetic state (idiohypophyseal diabetes) which is followed by a permanent diabetic state (metahypophyseal diabetes) is still unknown (1, 2, 3). All available evidence supports the hypothesis that somatotropin induces an increased peripheral need for insulin with a resultant increase in the production of insulin by the beta cells of the pancreas and that when the rate of insulin utilization exceeds the rate at which it can be manufactured, the beta cells undergo degranulation and hydropic degeneration. Thus, the idiohypophyseal diabetes is attributed to a relative insufficiency in the capacity of the pancreas to meet the increased peripheral requirements for insulin while the metahypophyseal diabetes which persists after the cessation of the somatotropin injections is attributed to the destruction of the beta cells (1, 2, 3).

The diabetogenic action of extracts of the anterior pituitary gland is inhibited in fasted, fat-fed, insulin-treated and phlorhizin-treated animals (4, 5, 6). These measures presumably reduce the peripheral requirements for endogenous insulin and thereby prevent the excessive stimulation otherwise produced by the administration of somatotropin. Since the hypoglycemic action of tolbutamide in most species is believed to be due, in part at least, to the secretion of insulin by the beta cells in the pancreas (7, 8, 9), it became of interest to evaluate the effect of this compound on the diabetogenic action of somatotropin.

METHODS AND RESULTS

Male dogs, weighing from 10 to 12 to 17 kilograms, were given a daily intramuscular injection of from 3 to 10 mgm. somatotropin per kilogram body weight for variable

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periods of time before, during and after the administration of tolbutamide. The tolbutamide was given daily by mouth in a single dose which varied from 50 to 100 mg. per kilogram body weight. All dogs were given a constant daily diet and any food that was not eaten by 4:30 p.m. was removed and weighed. Venous blood samples were taken between 8 and 9 every morning before the animals were fed or given any of the compounds under study. The daily urine excretion was collected quantitatively. The concentrations of glucose in the blood and the quantity excreted in the urine was determined by Nelson's method (10).

Illustrative of the effect of tolbutamide are the data depicted in Figure 1. As noted by others (1, 2, 3), within a few days after the start of the daily injections of somatotropin, the dog developed a marked hyperglycemia and glycosuria which persisted throughout the period during which somatotropin was given. By the third day after the daily injections of somatotropin were stopped, the blood sugar was back to the pre-treatment concentration and the glycosuria ceased. Subsequently, however, when the animal was given 50 mg. tolbutamide daily by mouth, the administration of the previously diabetogenic dosage of somatotropin produced only a small transient increase in the blood sugar concentration and a transient glycosuria.

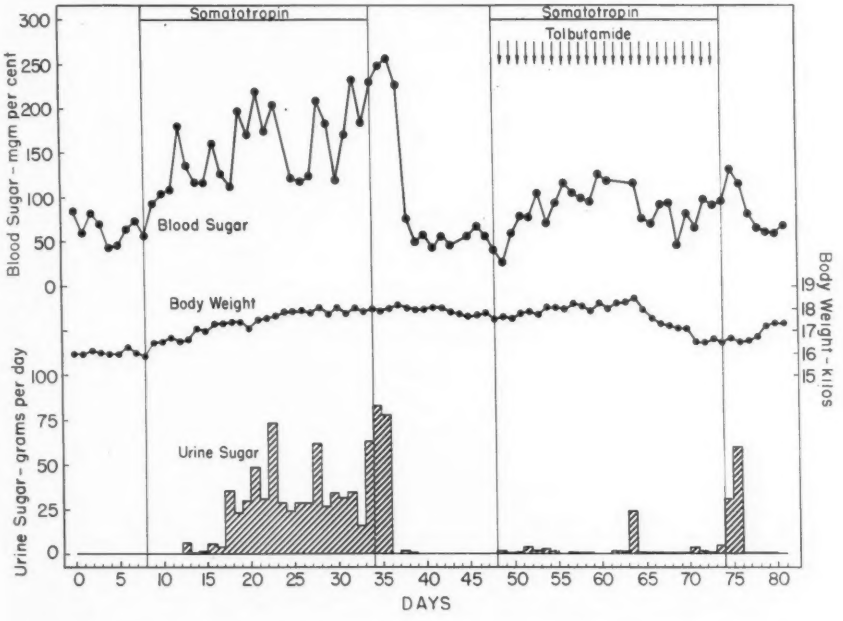

FIG. 1. Effect of tolbutamide on diabetogenic action of somatotropin. 5 mg. somatotropin per kilo injected intramuscularly daily during periods noted. During the second period of somatotropin administration, 50 mg. tolbutamide per kilo given daily by mouth.

The significance of these and similar data obtained with 8 other dogs is difficult to evaluate in view of the evidence that both somatotropin and tolbutamide produce beta cell stimulation. Yet tolbutamide produces a hypoglycemic response and somatotropin a diabetogenic response when given alone. The prevention of the diabetogenic response when both agents are given at the same time suggests that excessive stimulation of the beta cells of the pancreas may not be the principle determinant in the diabetogenic response to somatotropin.

Acknowledgments

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THE ACUTE EFFECT OF SALTS ON THE URINARY EXCRETION OF RADIOIODIDE IN MICE[1]

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ABSTRACT

The acute effect of various ions on the urinary excretion of radioiodine was investigated by measuring the radioiodine excretion in the urine of mice injected with I^{131} subcutaneously and given 0.517 milliequivalent of various salts through the gastric route.

Sodium, potassium, rubidium, chloride, bromide, iodide ions increased the excretion of radioiodine, while lithium, calcium, ammonium, carbonate and acetate ions did not. Glucose and water had no effect.

Since only those salts releasing one or more highly dissociated ions did increase the excretion of radioiodine, this effect appears to be due to an increased concentration of ions in the body.

ADMINISTRATION of sodium chloride to mice increases the urinary excretion of the iodide ion to an extent such that a relative iodine deficiency and a stimulation of the thyroid gland may occur (1). The chemical property in sodium chloride responsible for this effect was investigated in mice by administering sodium, chloride and other ions and measuring the amount of radioiodine excreted in the urine.

MATERIALS AND METHOD

The mice used were C_3H or albino of either sex, aged 2–9 months. The tables indicate the strain, sex and age used for each experiment. Prior to the experiment, they had been fed for 3 weeks on a low iodine diet composed of 89% corn meal, 10% dried brewer's yeast and 1% calcium carbonate and containing only traces of sodium and chloride (2), to minimize the urinary excretion of iodide in controls. Then the animals were given 0.2 ml. of an aqueous solution containing 0.517 milliequivalent of whatever salt was under investigation (30 mg. in the case of sodium chloride). The controls received an equal volume of water. The solution—or water—was introduced into the stomach either by tubing, using a polyethylene tube "intramedic" No. 10, the external diameter of which was 0.6 mm., or by injection through the muscular wall of the stomach. Immediately afterwards, the mice received a subcutaneous injection of 10 μc. carrier free I^{131} in 0.2 ml. water. Each animal was then placed in a 250 ml. beaker fitted with a wire mesh floor allowing the urine to settle at the bottom and was given access to distilled water

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but not to food. Two and a half hours later, the mouse was anaesthetized with ether in the beaker. Bladder and thyroid gland were removed. The thyroid was homogenized in 10 ml. 2N NaOH. The beaker containing the urine and the bladder were washed with 5 ml. 2N NaOH and 10 ml. water. In both cases 1 ml. of the solution was poured on a watch glass-supported lintine filter disc, dried at room temperature and counted with a Geiger-Müller counter. Standards were made for thyroid and urine by adding a known amount of radioiodine to sodium hydroxide solutions. The counts were corrected for background and expressed as a per cent of the injected dose of radioiodine.

In one experiment the renal clearance of radioiodine from the blood was measured. Two groups of mice were treated as indicated above, except that one group was sacrificed after 1 hour and the other after 3 hours. In addition, the radioiodine content of blood (obtained by heart puncture) at sacrifice time, was measured. The clearance over the 2-hour interval between the 1st and 3rd hour after the beginning of the experiment was calculated according to the formula

$$U_3 - U_1 \cdot \frac{\text{Ln}B_3 - \text{Ln}B_1}{B_3 - B_1} \cdot \frac{1}{120} \text{ ml. cleared per minute,}$$

where U_1 and U_3 are the amount of I^{131} excreted in urine during the first hour and the first 3 hours respectively, and B_1 and B_3 the I^{131} concentrations in blood at 1 and 3 hours respectively.

RESULTS

Lithium chloride increased the urinary excretion of radioiodine (Table 1). The effect of this salt may be attributed to the chloride ion since three other lithium salts (carbonate, acetate and fluoride) did not significantly influence this excretion.

The two sodium salts tested, namely sodium phosphate and sodium bicarbonate increased the urinary excretion of radioiodine (Table 1). The effect of sodium bicarbonate can be attributed to the sodium ion since neither the carbonate ion of lithium carbonate nor the hydrogen ion of acetic acid had any effect. Thus both chloride and sodium ions were efficient in increasing iodide excretion. However, when used separately, neither one nor the other of these ions increased radioiodine excretion to the same extent as when used jointly in sodium chloride.

Several salts containing neither sodium nor chloride ions were also capable of influencing iodide excretion (Table 1). Thus, rubidium carbonate increased it, presumably through the rubidium ion. Potassium bicarbonate increased iodide excretion ten times, but the effect could not be proved statistically due to the small numbers of survivors. Ammonium carbonate had no effect, calcium acetate and acetic acid decreased radioiodine excretion. All halogenides tested except fluoride increased radioiodine excretion. Iodide was significantly more potent than the other halogenides. Glucose had no influence on this response.

Administration of large amounts of water had no effect. Table 1 shows the results obtained in animals given food and water up to the time of injection, but other groups denied access to water or both food and water during the night preceding the experiment did not show any effect of water

TABLE 1. THE ACUTE EFFECT OF 0.517 MILLIEQUIVALENT OF VARIOUS SALTS ON THE URINARY EXCRETION AND THYROID UPTAKE OF RADIOIODINE IN MICE

Treatment	Number of mice	% of injected radioiodine in	
		Urine Mean ± S.E.	Thyroid Mean ± S.E.
(C_3H male 2 months-old mice)			
Control	8	3.7 ± 1.2	23.1 ±2.8
Lithium carbonate (Li_2CO_3)	7	5.5 ± 1.6	*7.9 ±4.5*
(Albino male 4 months-old mice)			
Control	8	9.9 ± 2.3	12.7 ±1.6
Lithium acetate	7	13.7 ± 5.5	9.1 ±1.0
(C_3H male 2.5 months-old mice)			
Control	6	4.7 ± 1.1	26.8 ±3.1
Sodium bicarbonate ($NaHCO_3$)	7	*37.2 ± 4.6*	23.4 ±2.9
Di-sodium phosphate (Na_2HPO_4)	7	*33.6 ± 4.9*	19.7 ±1.2
Sodium chloride	7	*53.8 ± 3.6*	*17.1 ±1.4*
(C_3H male 5 months-old mice)			
Control	8	3.2 ± 0.9	23.4 ±1.7
Potassium bicarbonate ($KHCO_3$)	3	30.9 ±15.2	*10.8 ±2.6*
Sodium chloride	8	*56.2 ± 2.8*	*13.5 ±1.2*
(Albino male 3 months-old mice)			
Control	8	12.7 ± 3.1	11.8 ±0.7
Rubidium carbonate (Rb_2CO_3)	8	*38.9 ± 2.6*	11.0 ±2.0
Sodium chloride	8	*47.8 ± 3.8*	10.4 ±1.2
(Albino male 4 months-old mice)			
Control	8	9.9 ± 2.3	12.7 ±1.6
Ammonium carbonate $(NH_4)_2CO_3$	3	16.0 ± 5.0	13.1 ±4.2
Sodium chloride	7	*50.2 ± 5.3*	14.4 ±2.3
(C_3H male 5 months-old mice)			
Control	8	3.2 ± 0.9	23.4 ±1.7
Calcium acetate	7	*1.1 ± 0.3*	*9.7 ±1.5*
Acetic acid	8	*0.4 ± 0.1*	*7.5 ±1.5*
(C_3H female 9 months-old mice)			
Control	8	3.1 ± 0.6	10.4 ±3.6
Lithium fluoride	7	6.9 ± 2.4	11.4 ±5.3
Lithium chloride	7	*14.1 ± 2.5*	12.3 ±4.4
Lithium bromide	8	*17.3 ± 2.4*	11.0 ±4.6
Lithium iodide	8	*38.5 ± 5.0*	*0.14 ±0.04*
Sodium chloride	8	*55.6 ± 2.8*	7.1 ±3.2
Glucose*	8	3.4 ± 1.0	8.5 ±2.3
(C_3H male 3.5 months-old mice)			
Water 0.1 ml.	8	3.1 ± 0.7	15.3 ±2.7
Water 1.5 ml.	8	2.7 ± 1.0	15.7 ±1.3

The italicized values are significantly different from the respective controls.
* 0.517 millimoles.

either. (After injection of water, the animals were not allowed to drink water.)

The thyroid uptake of radioiodine was unchanged after administration of most of the salts, and was occasionally slightly diminished. But in mice given iodide, the thyroid uptake was drastically reduced and was only 1/79th of the uptake in controls.

A number of salts were found to be toxic. The toxicity of potassium bicarbonate and ammonium carbonate is reflected by the small number of animals surviving after 2.5 hours (Table 1). Hydrochloric acid, ammonium chloride, ammonium fluoride, sodium fluoride, potassium fluoride, were also tested, but were too toxic for conclusions to be reached regarding iodine excretion.

To investigate whether the increased excretion of radioiodine after administration of sodium chloride is due to a change in the functional activity of the kidney, the radioiodine clearance from blood was measured between the first and third hours following treatment. Table 2 shows that in sodium chloride treated mice the clearance was raised to 22 times the value in controls.

DISCUSSION

The respective roles of sodium and choride ions in raising the urinary excretion of iodide are clearly pointed out by the data obtained: both sodium and chloride ions increased the radioiodide excretion in urine (Table 1) and, therefore, both are responsible for the effect observed after administration of sodium chloride.

TABLE 2. EFFECT OF SODIUM CHLORIDE ON BLOOD RADIOIODIDE CLEARANCE BY THE KIDNEY

Treatment (Albino male 3 months-old mice)	Sacrifice time (hours)	Number of mice	% of injected I^{131} in		Blood I^{131} kidney clearance (ml./minute)
			Urine	1 ml. blood	
			Mean ±S.E.	Mean ±S.E.	
Controls	1	7	7.9±2.1	3.8±0.4	0.007
	3	7	11.2±2.8	3.7±0.5	
Sodium chloride, 30 mg.	1	7	17.4±4.3	3.0±0.5	0.152
	3	7	50.8±5.9	1.0±0.4	

The data obtained indicate that a similar effect could also be produced by ions other than sodium and chloride. However, the fact that some salts were not effective raised the question whether any particular chemical property is responsible for the effect. When the salts investigated were divided into those that do and those that do not increase iodine excretion, it appeared that the effective salts were those which introduced a large number of free ions in the body. As is well known, a well defined fraction of any salt in solution dissociates into ions. Thus, a large proportion[2] of sodium, potassium, rubidium, chlorine, bromine and iodine ions dissociate from the other moiety in the salts used. All of them increased the excretion of radioiodine. On the contrary, the salts formed from a poorly dissociated

base[2] (e.g. ammonia) and a poorly dissociated acid[2] (e.g., hydrofluoric, carbonic, acetic), and which, therefore, liberate few ions, were inactive. Bases well dissociated like lithium and calcium hydroxides,[2] but which form poorly soluble salts with physiological anions (phosphate, carbonate)[3] were also inactive. The importance of the ionic concentration is also pointed out by the fact that sodium chloride which releases in solution 2 well dissociated ions was more potent than sodium bicarbonate or lithium chloride, which introduce only one free ion, respectively sodium or chloride. In addition, glucose whose ionization is negligible did not influence iodine excretion. Thus, it may be concluded that the urinary excretion of iodine is increased whenever the concentration of ion in the body increases. This view also accounts for the observation that physiological saline has little effect on the excretion of radioiodine (3).

Administration of iodide-I^{127} (as lithium iodide), in addition to raising the ionic concentration of plasma like other salts, has two effects which do not occur under treatment with the other salts. Firstly, the iodide ion which is administered with lithium iodide behaves exactly like the tracer which is being measured, and therefore "dilute" it. Secondly, in the presence of a pharmacological amount of iodine such as that administered (66 mg.), the metabolism of iodide (and therefore of radioiodide) may be modified not only quantitatively but also qualitatively.[4] The respective role of these three factors in increasing radioiodide excretion is not known. It is possible that the increase in plasma ionic concentration raises the excretion of radioiodide to the same extent as in lithium chloride or bromide treated mice, and that a further raise is produced by the "dilution" factor, by saturation of the iodide reabsorption capacity in the kidney tubule.

The mechanism by which sodium chloride increases the urinary excretion of radioiodide cannot be explained merely on the basis of changes in the concentration of plasma radioiodide resulting from alterations in the extrarenal metabolism of iodine (e.g. change in blood circulation or in inter/intracellular distribution) but involves a change in the functional activity of the kidney, since the clearance of the isotope was sharply increased (Table 2). This is in agreement with earlier observations (1) and with the increase in radioiodine clearance after administration of a number of sodium salts (chloride, bromide, iodide, bicarbonate) as well as of choline iodide reported by Halmi *et al.* (4).

[2] Degree of ionization of normal solutions of NaOH: 0.73, KOH: 0.77, HCl: 0.784, NH_4OH: 0.004, HF: 0.070, H_2CO_3: 0.0017, CH_3COOH: 0.004, LiOH: 0.63, $Ca(OH)_2$ (N/16): 0.90 (5).

[3] Solubilities in cold water of Li_3PO_4: 0.039 gm./100 ml., $Ca_3(PO_4)_2$: 0.002 gm./100 ml., and $CaCO_3$: 0.0015 gm./100 ml. (5).

[4] Such a change has been observed in thyroxine metabolism (6): normally excreted in feces only, the hormone appeared in the urine after injection of large doses.

Effect of salts on radioiodine uptake by the thyroid

The occasional diminution in thyroid uptake observed after administration of salts may be attributed to various factors. In the case of compounds which do not increase the urinary excretion of radioiodine, such as lithium carbonate, calcium acetate and acetic acid, the diminution in thyroid uptake is probably due to the toxicity of the salts. This factor probably also accounts for the diminution of radioiodine excretion in calcium acetate and acetic acid treated mice. In animals given sodium chloride the occasional decrease in thyroid uptake may be due to urinary losses depriving the thyroid of radioiodine although toxicity may also play a role. The decrease in thyroid uptake reflects the decrease in blood radioiodine level. Both decreases are slight early after treatment as in these experiments, but are much more marked later on (1). The very low uptake observed in mice treated with lithium iodide comes from the I^{127} load which dilutes I^{131} and saturates the capacity of the thyroid to incorporate iodine into organic compounds.

In conclusion, the fact that only highly dissociated salts increased the urinary excretion of radioiodine, suggests that iodide excretion is influenced by the ionic concentration of plasma.

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DISTRIBUTION OF C^{14}-ESTRONE IN RATS AND DOGS[1] BEFORE AND AFTER PLASMAPHERESIS

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ABSTRACT

The present study was undertaken to demonstrate possible effects of plasmapheresis on the distribution and clearance of C^{14}-estrone in rats and dogs. Plasmapheresis was accomplished by removing $\frac{1}{3}$ of the animal's blood volume, substituting dextran or polyvinyl pyrrolidone solution for the plasma, and reinjecting the mixture. This procedure was repeated 5–6 times. The plasma protein concentration fell to less than 10% of the starting levels. In dogs following plasmapheresis the clearance of unconjugated radioactivity from the plasma did not differ materially from that in the same dogs before plasmapheresis. On the other hand, the glucosiduronate levels in the plasma were much higher following plasmapheresis than in the control experiments. Approximately twice as much radioactivity, primarily as glucosiduronates, appeared in the urine following plasmapheresis as before. In rats, distribution of radioactivity was determined in organs, excreta and blood 20 minutes following the injection of C^{14}-estrone. In control rats 84% of the radioactivity appeared in the gastrointestinal tract and liver (54% and 30%, respectively) compared to 42% (15% and 27%, respectively) in the rats following plasmapheresis in which the plasma was replaced with 6% dextran in saline. Interestingly, 25% of the radioactivity was found in the lungs of the rats after plasmapheresis as compared to 2% in the controls. The administration of polyvinyl pyrrolidone to rats following plasmapheresis resulted in the appearance of 41% of the radioactivity in the gastrointestinal tract and liver and 6% in the lungs. More radioactivity was present in some of the organs and fat of rats who had undergone plasmapheresis and given polyvinyl pyrrolidone than in the control rats or in the rats given dextran.

THE binding of estrogens to plasma proteins has been amply demonstrated (1), but the significance of this binding in the transport, distribution, or metabolism of these steroid hormones has not been ascertained. The study reported here represents an attempt to answer some of these problems and is based on the distribution of injected C^{14}-estrone in normal rats and dogs before and after plasmapheresis.

MATERIALS AND METHODS

Male and female Sprague-Dawley rats (250–425 gm. in weight) were anesthetized with sodium pentobarbital (25 mg. per kg.) given intraperitoneally. The carotid artery

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and jugular vein were cannulated with small polyethylene catheters. Seven to ten ml. of blood were removed with a syringe from the arterial cannula, placed into a graduated centrifuge tube containing 0.1 mg. heparin, and centrifuged for 10 minutes. The plasma was removed and replaced by an equivalent volume of 6% dextran in 0.15M saline (Abbott) or 3.5% polyvinyl pyrrolidone (PVP-Macrose).[2] The mixture was reinjected into the jugular vein through the venous catheter. In the rats undergoing plasmapheresis this procedure was repeated five times. Plasmapheresis was accomplished in 1–2 hours. At the end of the plasmapheresis procedure 16-C^{14}-estrone (4,000,000 counts per minute /mg.) in 1 ml. of 6% dextran or PVP-Macrose was injected through the venous cannula and the cannula washed through with a small amount of plasma substitute. Each rat was injected with 125,000 counts per minute. The rats were sacrificed by exsanguination through the arterial catheter twenty minutes following the injection of the steroid. The organs and tissue specimens were then removed, blotted free of blood, and stored at −10° C until homogenized. The frozen tissues were weighed and homogenized in water either by hand or mechanically, and extracted three times with large volumes of absolute ethanol. The latter was evaporated by air to a small volume, part of which was plated and the radioactivity counted. Digestion of the protein residue with sodium hydroxide (0.1N) followed by extraction with chloroform failed to reveal any residual radioactivity. Control animals were operated on similarily, blood withdrawn, centrifuged and then reinjected. To some of these bloods concentrated solutions of dextran were added to produce a final concentration in the plasma of 2–6%, to others 90 mg. or less of dextran were added.

Plasmapheresis was performed in dogs anesthesized with 25 mg./kg. of sodium pentobarbital I.V. by removing 300–500 ml. of blood from the femoral artery, centrifuging the blood, and substituting the plasma with 6% dextran in saline. This procedure was repeated 5 to 6 times. The cells were reintroduced by intravenous infusion through the jugular vein. The dogs were then injected intravenously with 0.5–1.0 μc. of C^{14}-estrone (1 μc. per 370 g.).

The methods for extraction and hydrolysis of the plasma, urine, and erythrocytes with β-glucuronidase, at pH 1, and hot acid, and the determination of radioactivity in the urine and blood have been previously described (2). Total plasma proteins were determined by the method of Gornall *et al.* (3). The electrophoretic pattern was determined by free zone electrophoresis using a Perkin-Elmer Model 38 electrophoresis apparatus. All samples were dialyzed to equilibrium and the determination performed in barbiturate buffer (pH 8.6, ionic strength 0.1) according to standard techniques.

RESULTS

Since the major aim of the present study was to obtain as low a level of plasma protein as was possible, the most critical part of the experiment concerned itself with the rate of the plasmapheresis and the selection of a substitute for the removed plasma. Previous experiments had shown that the production of plasmapheresis in dogs over a prolonged period of time (one week) did not result in sufficient lowering of the plasma protein level. Hence, plasmapheresis was performed over a period of one to two hours, after which time the animals were injected with the radioactive estrogen.

[2] PVP-Macrose was kindly supplied by Dr. P. Puetzer of Shenlabs Pharmaceuticals, Inc. It consisted of a solution of 3.5% PVP containing 0.65% NaCl, 0.042% KCl, 0.025% $CaCl_2$, 0.0005% $MgCl_2$, and 0.024% $NaHCO_3$.

In all experiments the plasma protein concentration fell to less than 10% (0.3–0.6 gm.%) of the original level (Fig. 1).

Initial studies showed that the replacement of plasma with physiological saline resulted in early death of the animals. Hence, in the experiments in dogs the plasma was replaced by 6% dextran in saline. The condition of the animals was good throughout the time of the experiments. Figure 2 shows the clearance of the mean unconjugated and conjugated radioactivity from the plasma of five dogs before and after the plasmapheresis, following the injection of C^{14}-estrone. The dogs served as their own controls. A period of at least three weeks elapsed following injection of radioactive estrogen be-

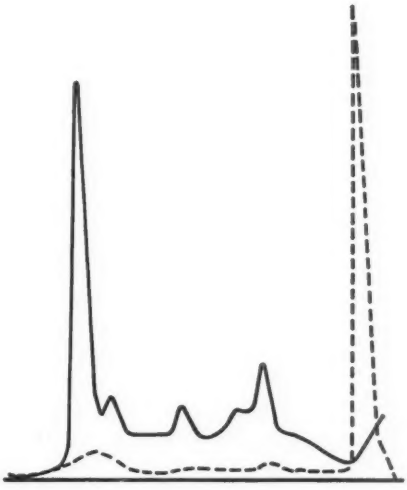

FIG. 1. Electrophoresis pattern of plasma of Dog No. 2 before (solid line) and following (broken line) plasmapheresis. High peak on the right following plasmapheresis represents the injected dextran. Note great reduction in the amount of protein following plasmapheresis.

fore the plasmapheresis experiments were performed. The conjugated radioactivity was maintained at higher levels following plasmapheresis than in the control experiments. The conjugated radioactivity was primarily in the form of glucosiduronates.[3] This finding assumes further significance when examined in the light of the urinary data shown in Figure 3. The amount of radioactivity excreted in the urine during the six hours following injection of C^{14}-estrone was much higher following the plasmapheresis experiments than during the control period, in spite of the fact that the amount of urine produced following plasmapheresis was smaller than during the control period. As in the plasma, the major portion of the hydrolyzable and extractable radioactivity in the urine was in the form of glucosiduronates. Moreover, following plasmapheresis the amounts of the

[3] The term "glucosiduronates" has been used in this paper to refer to steroid metabolites conjugated with glucuronic acid. This nomenclature is in keeping with that published for "Rules of Carbohydrate Nomenclature," Chem. Eng. News, **31**: 1776, 1953.

TABLE 1. MEAN AND RANGES (IN PARENTHESES) OF PERCENTAGES OF INJECTED DOSE OF C^{14}-ESTRONE IN ORGANS OF RATS BEFORE AND AFTER PLASMAPHERESIS

Organs	Group A Control rats No plasmapheresis (3M +2F)	Group B Plasmapheresis plus dextran substituted for plasma (4M +2F)	Group C Dextran added to plasma, No plasmapheresis (2M +2F)	Group D Plasmaphersis plus PVP substituted for plasma (6M)
G-I tract plus liver	84% (80–89)	42% (18–64)	66% (54–80)	41% (31–48)
G-I tract	54% (43–71)	15% (8–26)	33% (22–43)	18% (6–25)
Liver	30% (15–38)	27% (10–38)	33% (15–52)	23% (15–37) (35%)*
Lung	2.0% (1–2.5)	25% (6–46)	7.0% (4.5–20)	6.0% (2–15)
Kidneys	1.4% (1.1–1.6)	2.3% (1.9–4.6)	2.8% (2.2–5.0)	3.5% (3.0–4.8)
Heart	0.3% (0.2–0.5)	1.4% (1.1–2.6)	0.8% (0.6–1.6)	1.8% (1.4–2.9)
Testes	1.4% (1.1–2.6)	0.8% (0.4–1.8)	1.6% (1.0–2.0)	1.7% (1.3–3.1)
Spleen	0.2% (0.1–0.3)	0.4% (0.3–0.7)	0.4% (0.3–0.5)	0.3% (0.1–0.5)
Adrenals	0.1% (0.09–0.12)	0.2% (0.1–0.4)	0.2% (0.1–0.4)	0.1% (0.09–0.12)
Bladder and urine	0.5% (0.3–0.7)	0.2% (0.1–0.4)	0.7% (0.4–1.1)	0.3% (0.2–0.6)
Uterus and adnexae	0.4% (0.2–0.6)	0.9% (0.7–1.1)	0.7% (0.3–1.4)	—
Muscle (c/m/g.)	253	163	245	231
Fat (c/m/g.)	476	515	348	796

* Percentage of injected radioactivity in the livers of two additional rats not shown in the Table.
M = male.
F = Female.
c/m/g = counts per minute per gram tissue.

urinary conjugated C^{14}-estrone metabolites were much higher than those present in the control urines.

Plasmapheresis in the rats presented several problems, primarily with regard to the choice of the plasma substitute. Examination of Table 1 reveals several interesting points. In the control animals (Group A) 84% of the injected radioactivity was present in the liver and the gastrointestinal tract 20 minutes after the injection of C^{14}-estrone. Following plasmapheresis in rats in which 6% dextran in saline was substituted for the

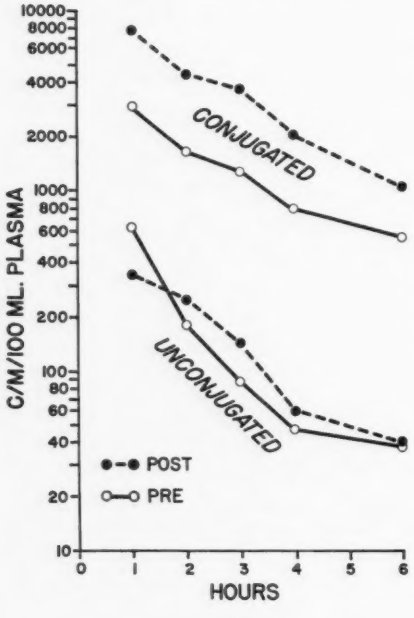

FIG. 2. Clearance of mean unconjugated and conjugated radioactivity (following the i.v. injection of C^{14}-estrone) from the plasma of dogs before and after plasmapheresis. Note much higher levels of radioactivity in conjugated (glucosiduronate) fraction following plasmapheresis.

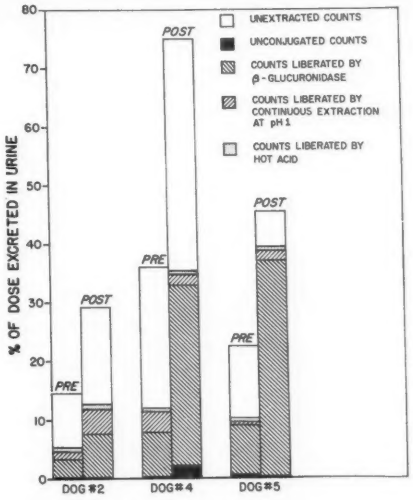

FIG. 3. Excretion of radioactivity, (following the i.v. injection of C^{14}-estrone) in the urine of 3 dogs before and after plasmapheresis.

plasma (Group B), the total amount of radioactivity present in the liver and the gastrointestinal tract was on the average only 42% (27% in the liver and 15% in the gastrointestinal tract). An extraordinary amount of radioactivity was present in the lungs (25%), with values as high as 46%. In the control rats only 2% of the radioactivity was present in the lungs.

It was important to ascertain whether the above findings were due to the lack of plasma proteins or a special effect of dextran (4, 5). Others have reported that in animals receiving dextran in saline as a substitute for the plasma the mortality rate was high, with most of the animals dying of respiratory of circulatory failure. Some respiratory embarrassment was present in our experiments in some of the rats that survived and in which dextran was used as a plasma substitute following plasmapheresis. Such rats in our experiments received from 600 to 1000 mg. of dextran. To test the effect of dextran without plasmaphoresis, a solution of dextran was concentrated three fold in a freeze-drying apparatus. This concentrated solution (0.5–3.0 ml.) containing 90–540 mg. was added to the centrifuged blood of normal rats before it was injected. It should be pointed out that the plasma was not removed from these bloods and that the cells were resuspended by gentle shaking of the sample before injection. The averaged results, Table 1 (Group C), show that 7% of the radioactivity was found in the lung. In one rat, who had received 540 mg. of dextran, 20% of the radioactivity was present in the lung, whereas in another identically treated rat over 4.5% was present in the lungs.

In order to obviate the untoward effect of dextran, plasmapheresis was performed on rats and the plasma replaced with PVP-Macrose. The latter is considered to be less toxic in the rat than dextran (4, 5). The results are shown in Table 1 (Group D). The rats seemed to tolerate PVP-Macrose

better than dextran. The average radioactivity found in the gastrointestinal tract and the liver of these animals was 41% with approximately 30% in the liver. About 6% of the radioactivity was found in the lung, with the range being 1–15%. More radioactivity was found in the heart, kidneys and fat of the rats receiving PVP as a plasma substitute than in any other group.

The distribution of the radioactivity in the plasma and erythrocytes following the injection of C^{14}-estrone in rats showed some deviation from the controls when plasma substitutes were added (Table 2). More of the radio-

TABLE 2. MEAN RADIOACTIVITY IN PLASMA AND ERYTHROCYTES OF RATS 20 MINUTES FOLLOWING THE INJECTION OF C^{14}-ESTRONE (c/m/10 ml.)†

Group*	Unconjugated fraction	Glucosiduronate fraction	"Sulfate" fraction	Acid hydrolyzable fraction	RBC
A	460	300	120	100	680
B	1100	150	100	20	1100
C	900	300	110	50	1300
D	1100	80	100	110	1100

* Groups A, B, C and D are identical with those in Table 1.
† The radioactivity was measured in samples of blood the volume of which varied from 7–10 ml.

activity was present in the unconjugated fraction and associated with the red blood cells in animals receiving plasma substitutes than in the control rats. The total amount of radioactivity present in the total blood volume of the largest rat at the time of exsanguination represented less than 10% of the injected radioactivity.

No binding of C^{14}-estrone could be demonstrated to 6% dextran in saline or PVP-Macrose under equilibrium dialysis techniques previously described (1).

DISCUSSION

From Figure 2 it is evident that plasmapheresis of dogs had no effect on the concentration or rate of disappearance of unconjugated radioactivity following the injection of C^{14}-estrone, but did produce an increase in the level of the conjugated fraction. The reason for this dichotomy is not known and exemplifies the need for more detailed knowledge of the binding of conjugates than previous experiments have afforded (1, 8). The greater urinary excretion of radioactivity following plasmapheresis is thought to be a reflection of the higher plasma levels of the conjugated steroids rather than an effect of the lowered protein concentration, since the rate of disappearance of the conjugated radioactivity was nearly the same as that before plasmapheresis. This would be consonant with the high binding capacity of albumin for the estrogen conjugates (8). Even at the low concentration of plasma protein attained following plasmapheresis, there were probably several grams of albumin left in the circulation.

Previously reported studies from our laboratory indicated a possible

relationship between the binding of a steroid to human serum albumin and its excretion in the bile of human subjects (9). Those steroids which are bound avidly to serum albumin (estrogens, progesterone) are primarily excreted in the bile, while those steroids which are weakly bound by human serum albumin (cortisol, 11β-hydroxyandrostenedione) are not excreted in the bile to any great extent. If the binding to dog serum albumin bears a similar relationship between the steroids and the excretion in the bile, the plasmapheresis and the resultant lack of serum albumin could conceivably result in decreased biliary excretion of the radioactive metabolites of C^{14}-estrone. It should be pointed out that plasmapheresis may remove factors other than serum proteins from the plasma of the dogs which may be necessary for the normal excretion of the metabolites of estrone in the bile. Thus, the changes in distribution may be related more to these factors than to the plasma proteins.

Even though the period of study following injection of the steroid was very brief (20 minutes), it can be seen that in the control rats 80–89 per cent of the radioactivity was already present in the liver and gastrointestinal tract, with 43–71 per cent in the latter. Valcourt *et al.* (6) have shown that following intramuscular or intragastric administration of C^{14}-estrone to rats 70% of the radioactivity appeared in the stools in 4–5 days. Following cannulation of the bile duct, 85% of the radioactivity appeared in the bile, with only negligible amounts in the urine or feces. Studies in our laboratory have shown that following intravenous injection of C^{14}-estrone into rats with bile ducts cannulated, over 90 per cent of the radioactivity is recovered in the bile within one hour (7). The data of the present study also indicate rapid excretion of the radioactivity into the gastrointestinal tract, probably via the bile. The liver contained considerable amounts of radioactivity and, on the basis of the studies mentioned previously (6, 7), there is reason to believe that these metabolites were destined to be excreted in the bile. The endocrine glands examined contained a low level of radioactivity, as did the bladder and its contents of urine, when compared to that of the liver. It is interesting to note that the fat contained more radioactivity per gram of tissue (approximately 500 counts per minute) than the muscle (250 counts per minute). Negligible amounts of radioactivity were present in the blood, most of it appearing in unconjugated fraction of the plasma or associated with the red blood cells.

Since the administration of dextran, and also probably PVP-Macrose, in the rat results in adverse effects on the small blood vessels and other systems (4, 5), it is difficult to interpret the results of plasmapheresis. The presence of considerable radioactivity in the lungs of rats given dextran following plasmapheresis, and in some rats following PVP-Macrose, may be related to pooling, extravascular extravasation or trapping of the C^{14}-estrone by the reticuloendothelial system of the lung. The abnormalities in the distribution of the radioactivity following C^{14}-estrone injection in

rats given dextran or PVP may be related not only to the adverse effect of the plasma substitutes, but also to the lack of plasma proteins. Thus, dextran is known to adversely affect the vascular system of the rat (4, 5) and the abnormal distributions of the radioactivity of C^{14}-estrone may be accounted for on the basis of a shock-like state in the rats given dextran. This explanation does not hold true, however, for the rats given PVP. The reasons for the abnormal distribution of the radioactivity in these rats remain unknown.

It has been previously demonstrated that in the rat the biliary route serves as the major pathway for the excretion of steroid metabolites (6, 7, 10–14). Of all the steroids studied, C^{14}-estrone would seem to be the steroid most quantitatively excreted in the bile of the rat. The present studies indicate that the presence of plasma proteins plays an important role in the excretion of these metabolites in the bile of the rat and that in the absence of such proteins, the distribution of the injected radioactive C^{14}-estrone is modified with resultant decreased excretion of its metabolites in the bile.

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THE EFFECT OF ESTROGENS ON BONE PHOSPHATE ACCRETION, RESORPTION, AND EXCHANGE IN MICE

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ABSTRACT

The effect of estrone on the bone phosphate accretion rate, the size of the exchangeable fraction and resorption has been measured. The estrogen treatment enhanced both accretive and resorptive mechanisms associated with the non-exchangeable bone phosphate while there was little effect upon the size of the exchangeable phosphorus pool. The turnover time of stable bone mineral was reduced from 18.5 to 13.0 days by the treatment. An assay procedure in which an "accretion index" was calculated showed the relative potencies of estrone, estradiol benzoate and diethylstilbestrol to be in the ratio of 9:2:1, respectively.

IT IS an established fact that estrogens may modify bone growth and mineral deposition (1), but the extent to which these hormones effect such changes has not always been quantitatively elucidated. Recently Bauer *et al.* (2, 3) have described an analytical approach that makes it possible to measure separately the apparent accretive and resorptive mechanisms that exist simultaneously in bone. We have applied the formulae of Bauer *et al.* to a study of the effect of estrogen on bone phosphate accretion and resorption in mice. In addition, we have used an "accretion index" to define the relative potency of three estrogenic materials in terms of their ability to modify bone metabolism.

METHODS

Young, adult male mice fed a commercial laboratory chow were divided into 10 groups of 8 animals each. Five groups received 0.8 mg./kg. estrone in corn oil daily for 22 days, while the others received oil alone. At intervals of 4, 24, 48, 96 and 144 hours before sacrifice a treated and a control group were given subcutaneously 2 microcuries of P^{32} as sodium phosphate. All animals were sacrificed on the 23rd day and plasma and left tibiae were sampled. Plasma inorganic phosphate was estimated in the manner described by Bauer and Carlsson (2) using the colorimetic method of King (4). Aliquots of the acid filtrate of plasma were mounted directly on lens paper supported by copper planchets and the radioactivity measured with an end-window geiger tube. Tibia samples were autoclaved to facilitate removal of flesh and after cleaning were dried overnight at 110° C. The bones were weighed, dissolved in 1 ml. nitric acid and then 0.2 ml. perchloric acid was added to clarify the solution. Water was added to make 5 ml. and aliquots were mounted for radioactivity measurement as described above.

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To measure the effect of estrone on the apparent turnover rate of bone phosphate 2 groups of 16 male mice each were injected subcutaneously with 2 microcuries of radioactive sodium phosphate. The treated group then received daily 0.8 mg./kg. of estrone in corn oil; the control received the vehicle alone. Half the animals in each group were sacrificed after 7 days of treatment and the remainder after 14 days. Tibiae were analyzed for P^{32} as described above.

A simplification of the experimental approach described above provided a convenient assay procedure by which a comparison could be made of the effects of various estrogenic materials upon the accretive phase of bone metabolism. Estrone, estradiol benzoate, and diethylstilbestrol were administered daily in oil for a period of nine days to groups of 10 animals each. On the 5th day after the start of the treatment all animals including a control group received two microcuries of P^{32}; the mice were sacrified on the 10th day and plasma and tibiae were sampled. The measurements of plasma phosphate specific activity and of bone P^{32} were carried out as described above.

RESULTS AND DISCUSSION

Bone Phosphate Accretion, Exchange and Resportion. Table 1 illustrates the bone uptake of P^{32} and the specific activity of plasma inorganic phos-

TABLE 1. DISTRIBUTION OF P^{32} IN TIBIA AND PLASMA OF MICE TREATED WITH ESTRONE

Time (hrs.)	Plasma phosphate (μg./ml.)		Plasma specific activity* (% P^{32}/μg. P×10^3)		Tibia P^{32} (% P^{32}/gm.)	
	Control	Estrone	Control	Estrone	Control	Estrone
4	84.8	57.0	2.86	2.90	8.69 ± .39	10.5 ± .7**
24	58.6	51.7	2.04	1.74	9.47 ± .48	12.1 ± .7***
48	53.2	71.4	1.49	1.52	12.02 ± 1.14	12.2 ± .5
96	67.6	79.4	.769	.886	8.87 ± .32	11.6 ± .7***
144	71.5	75.5	.504	.577	8.73 ± .41	11.2 ± .4**
Mean	67.1	67.0	—	—	—	—

* Plasma specific activity is per cent P^{32} recovered per microgram plasma phosphate.
** $P < .05$.
*** $P < .01$; unstarred value is not significantly different from control.

phate as well as plasma inorganic phosphate concentration. Although there is a variation in plasma phosphate level between groups the average of the 40 treated mice is nearly identical to that of the 40 control animals.

For the calculation of the accretion rates, equation (7) of Bauer *et al.* (3) has been slightly modified.

$$\frac{P_{O4} - ATS_{M4}}{P_{O24} - ATS_{M24}} = \frac{S_4}{S_{24}} \qquad (1)$$

where: P_O is the per cent administered P^{32} recovered per gram bone at the time indicated (subscript); A is the accretion rate; T is the time after the administration of the isotope; S_M is the mean specific activity[1] of plasma

[1] Specific activity is defined as the per cent administered P^{32} recovered/μg. plasma phosphate.

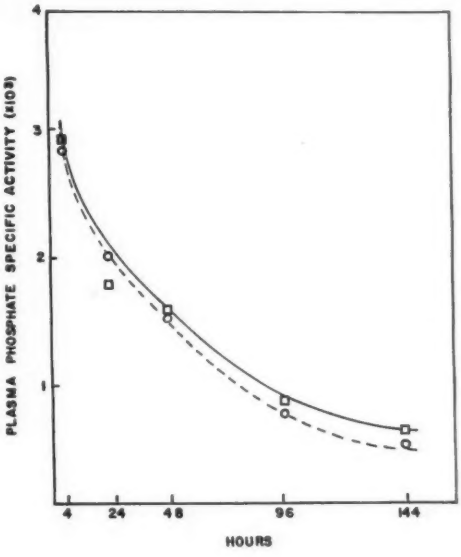

FIG. 1. Plasma specific activity-time curves. The abscissa indicates elapsed time after administration of isotope; the ordinate indicates specific activity defined as per cent P^{32} recovered per μg. plasma phosphate. Symbols: open circle, control; open square, estrone 0.8 mg./kg.

phosphate during this time interval; and S is the specific activity[1] of plasma phosphate at any given time. The integrated function, TS_M, was measured planimetrically from the specific activity-time curves for the plasma phosphate of the control and treated animals (Fig. 1), and P_O and S were taken from Table 1. Solving for A, the accretion rate[2], resulted in a value appreciably higher in the estrone treated animals than in the controls (Table 2). Since the value P_O in the estrone treated animals was in almost every case significantly different from that for the control animals, we assumed this difference in accretion rate to be statistically significant. To calculate E, the amount of P in the exchangeable fraction in bone, we have modified equation (4:1) of Bauer *et al.* (3) to read:

$$P_{O24} = ES_{24} + ATS_{M24} \tag{2}$$

where: E is the amount of P in the exchangeable fraction of bone; and the other symbols are as defined above. As shown in Table 2 the size of the exchangeable fraction of bone P was essentially unchanged by the estrone treatment. From these values for the rate of accretion of bone P and the size of the exchangeable P pool of bone we have calculated: P_A, the per cent administered P^{32} incorporated into the non-exchangeable fraction of bone through accretion,

$$P_A = ATS_M; \tag{3}$$

[2] During the interval 0–24 hours after isotope administration Bauer, *et al.* (3) demonstrated that resorptive mechanisms do not affect P^{32} levels in bone and therefore may be ignored in the calculation of "A" and "E" during this time interval.

TABLE 2. THE ACCRETION RATE AND THE EXCHANGEABLE FRACTION IN TIBIA OF MICE TREATED WITH ESTRONE

Treatment	Mean body wt. (gm.)	Accretion rate, "A" (μg. P/gm./hr.)	Exchangeable fraction, "E" (μg. P/gm.)
Control	32	82.5	2.56×10^3
Estrone, 0.8 mg./kg.	34	112.0	2.96×10^3

P_E, the per cent administered P^{32} present in the exchangeable fraction of bone,

$$P_E = ES; \tag{4}$$

and P_R, the per cent administered P^{32} removed through resorption,

$$P_R = P_A + P_E - P_O. \tag{5}$$

The bone fraction-time curves illustrating these values for control and estrone treated animals are shown in Fig. 2. The control pattern shown in this report is not unlike that presented by Bauer, *et al.* (2) for the rat. From the reports of these workers (2, 3) we have estimated a tibia shaft accretion rate of approximately 45 μg. P/hr./gm. in a 250 gm. rat, which is of same order of magnitude as our value of 83 μg. P/hr./gm. for the whole tibia of the mouse. It must be remembered that the accumulation of isotope in the shaft proceeds at a slower rate than in the ends of the long bones (3).

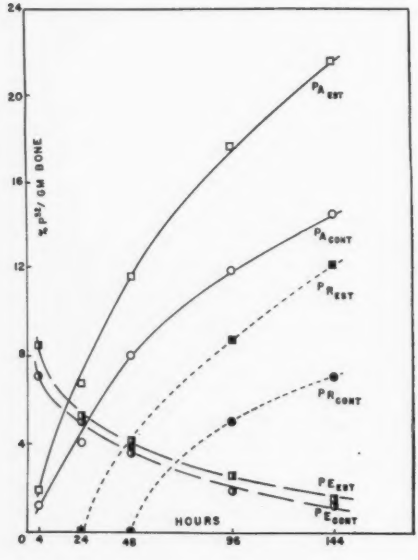

FIG. 2. Bone fraction-time curves. The abscissa indicates elapsed time after administration of isotope; the ordinate indicates per cent isotope calculated in bone fraction. Symbols: P_A, accretive incorporation of isotope into non-exchangeable fraction of bone; P_R, resorptive removal of isotope from bone; P_E, isotope present in exchangeable fraction of bone. Subscripts CONT and EST designate control and estrone treated groups, respectively.

It is evident that, whereas the amount of residual P^{32} in the exchangeable fraction (P_E) was only slightly increased by the treatment, both accretion and resorption mechanisms were stimulated to greatly increased activity by estrone.

Bone Phosphate Turnover. To confirm directly the evidence that bone phosphate turnover was enhanced by estrone treatment we have calculated the turnover time from the rate of disappearance of bone phosphate during the interval 7–14 days after isotope administration (Fig. 3). At this time

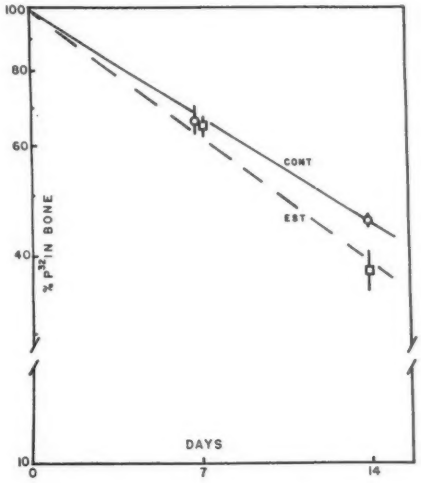

FIG. 3. Bone phosphate disappearance curves. The abscissa indicates the elapsed time after isotope injection; the ordinate shows the per cent P^{32} remaining in bone. The curves have been normalized so that the intercept at time 0 = 100%. The normalizing factor = 2.0. The vertical lines indicate the standard errors.

interval the amount of bone P^{32} contributed by the exchangeable fraction is very small (Fig. 2), and the observed decrease in bone P^{32} can be attributed to resorptive mechanisms associated with remodeling of the bone structure. Calculation of the turnover time of bone phosphate according to the method of Zilversmit *et al.* (8) resulted in a value of 18.5 days for the control animals while the estrone treated mice exhibited a turnover time of 13.0 days.

The evidence presented in this report indicates estrone augmented the turnover of bone phosphate in mice, both accretive and resportive mechanisms being stimulated to higher levels of activity than were observed in the control animals. Since Edgren and Calhoun (5) in similar experiments have shown estrone to induce an increase in bone density, we must conclude that to effect such a net increase in bone mineral phosphate the hormone must necessarily have enhanced bone mineralization more profoundly than bone remodeling.

Bone Accretion Index. To effect a quantitative comparison among various estrogenic materials an assay based on equation (3) described in the

TABLE 3. THE EFFECT OF ESTROGENS ON THE BONE ACCRETION INDICES OF MALE MICE

Experiment	Group	Compound	Dose (mg./kg.)	Plasma specific activity "S"†	Bone P^{32} "P_O"†	Accretion index "AI"†	Relative potency as determined by:	
							Accretion index	Bone density (5)
1	1	Control	—	.570	19.0	33.4±4.0		
	2	Estrone	0.1	.444	17.1*	38.6±1.7	—	—
	3	Estrone	0.4	.390	17.1*	43.8±3.3	—	—
	4	Estrone	0.8	.326**	17.8	54.6±4.4**	9.0	7.3
	5	Estradiol Benzoate	3.0	.410	21.8	53.1±4.7**	2.1	2.7
	6	Diethyl-stilbestrol	7.0	.355*	17.0*	47.8±2.2**	1.0	1.0
2	7	Control	—	.550	23.3	42.4±3.8		
	8	Estrone	0.5	.470	28.1*	59.7±3.8*	—	—
	9	Estrone	1.0	.473	29.7	62.8±5.5**	7.9	7.3
	10	Diethyl-stilbestrol	7.0	.542	30.2**	55.7±4.8*	1.0	1.0
	11	SC-6924***	50.0	.566	25.8	45.6±1.7	0	0.01

† Symbols are defined in the text, equation (6).
* $P < .05$; unstarred values are not significantly different from controls.
** $P < .01$.
*** SC-6924, Manvene, is 3 methoxy 16 methyl, 1, 3, 5(10) estratriene 16β 17β diol, and is an estrone derivative that profoundly modifies the lipid metabolism of the chick (6) but evidences only weak estrogenic properties.

preceding section has been developed. We have defined the accretion index as:

$$AI = \frac{P_O}{S} \tag{6}$$

where: AI is the accretion index; P_O is the per cent administered P^{32} recovered per gram bone; and S is the specific activity of plasma phosphate at the time of sacrifice. In arriving at this means of quantitatively expressing the effect of an estrogen upon bone metabolism we have ignored the effects of resorption and have assumed the plasma specific activity at T = 120 hours to be proportional to the integrated mean S_M. In eliminating consideration of resorption in the calculation we err on the conservative side, since we are, in effect, calculating a function of the *net* accretion rather than one of the total synthetic product. The similarity of shape of the plasma specific activity curves of the control and treated mice (Fig. 1) lends credence to our assumption that the plasma specific actvities at 120 hours are representative of S_M. We have in addition assumed the contribution of the exchangeable fraction, P_E, to the observed bone P^{32} at T = 120 hours to be negligible and the curves described in Figure 2 show this assumption to be essentially correct. As can be seen in Table 3 neither the plasma specific activity, S, nor the per cent P^{32} incorporated into bone, P_O, is as reliable a test of the ability of a material to enhance mineral deposition as is the accretion index. In two experiments using diethylstilbestrol, estradiol benzoate, and estrone a comparison of our potency estimates with those of Edgren and Calhoun (5), derived from bone density studies exhibited excellent agreement of the two methods.

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EFFECT OF CORTISONE ON GROWTH OF CHICK EMBRYOS DURING EARLY EMBRYOGENESIS[1]

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ABSTRACT

Cortisone acetate was administered on the initial day of incubation and at two or three day intervals thereafter to Cornish cross-White Hampshire chick embryos. The weight and length measurements were compared with controls on the fourth, seventh and tenth incubation days. A growth retarding effect of cortisone was demonstrated, which was more strikingly manifested in the alteration of the average length of the embryo in the earliest period, and in the alteration in average weight in the later periods. With this demonstration of growth retardation as early as the fourth day of incubation, it would appear unlikely that this effect is due to a suppression of somatotropin secretion by the hypophysis, since the latter is not fully differentiated prior to the tenth day. It would appear more likely that this effect represents a direct inhibition of protein synthesis and growth by cortisone.

RETARDATION of the growth rate and changes in the anatomy of the developing chick embryo following the administration of adrenal cortical extract (7), DCA (11), and cortisone acetate (3, 6, 13) have been reported. From these observations it would appear that there is a symmetrical retardation of growth. Karnosfsky *et al.* (6), in particular, have further reported that cortisone does not affect the growth rate until after the eighth day of development, and that the hormonal effect is diminished after the tenth day. They have postulated, therefore, that cortisone may interfere with some new substrate which normally appears during the midperiod of embryogenesis (eighth to twelfth days), coincident with the development of many new functions, including the peak development of the mesonephros.

Siegel *et al.* (13) have considered this cortisone effect as mediated through an inhibition of the pituitary, based primarily on the observation of Fugo (5) that hypophysectomy of the chick embryo has no appreciable effect on its growth and development until after the tenth day of incubation, and by analogy from the observations on postnatal animals of various species (2, 4, 12, 14), which show cortisone effects having a hypophyseal depend-

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ency. Furthermore, Allbright (1) has considered the well-known suppression of protein synthesis by cortisone an "anti-anabolic" rather than a direct catabolic effect, and Li (8) is of the opinion that this effect is one of an antagonism to hypophyseal growth hormone. On the other hand, Buno and Goyena (2) have shown a direct *in vitro* inhibition of the growth of chick embryo femur by cortisone. The present experiments compare the growth retardation of cortisone on the chick embryo prior to day 4, before the development of an intrinsic functional endocrine system, with that obtained by days 7 and 10 when there is doubtful intrinsic endocrine activity.

MATERIALS AND METHODS

Cornish-cross, White Hampshire chick eggs were drilled with a sterile teasing needle and injected chorioallantoically with the various solutions and suspensions noted below, using a 1 ml. syringe. In the first experiment the embryos received injections on the

TABLE 1. INJECTED ON DAYS 0, 3, 6 AND 9: EXAMINED ON DAY 10

Experiment	Dose	Survival rate	Average weight	Average length
Cortisone Acetate	1 mg. in 0.2 ml.	13/18	$1.27 \pm .14_1$* gm. (1.05–1.55)**	27.6 ± 1.5* mm. (25.0–30.0)**
Cholesterol	1 mg. in 0.2 ml.	10/18	$1.64 \pm .09$ gm. (1.45–1.75)	30.2 ± 2.8 mm. (21.0–30.5)
Sodium Chloride	180 mg. in 0.2 ml.	12/18	$2.93 \pm .13$ gm. (2.65–3.20)	32.8 ± 0.5 mm. (32.0–33.5)
Sham Operated		15/18	$2.73 \pm .14$ gm. (2.50–3.0)	34.6 ± 0.5 mm. (33.5–35.5)

* Standard deviation.
** Range of variation.

day of incubation, and on the third, sixth and ninth days thereafter. In the second experiment injections were made on the day of incubation, and on the second, fourth and sixth days thereafter. And in the third experiment the embryos were injected on the day of incubation and on the second day thereafter. In the first two experiments the embryos were examined on the day following the last injection, and in the third experiment on the fourth incubation day. In each experiment, all groups were injected at the same time and incubated simultaneously.

One group in each experiment received 1 mg. of cortisone acetate in 0.2 ml. of distilled water per injection; another group in each experiment received 0.2 ml. of 0.9% saline per injection, and a third group was sham operated. A cholesterol suspension (1 mg. in 0.2 ml. distilled water) was used as an additional control in the first experiment, but this was replaced by an aqueous solution of sodium desoxycholate in experiments 2 and 3, because of the latter's closer chemical identity with cortisone and because the insolubility of cholesterol presented difficulties in maintaining a uniform suspension. Thus each experiment consisted of 3 control groups and a cortisone test group.

At the completion of the incubation period, the embryos were separated from all extraembryonic materials, blotted thoroughly and weighed individually. They were also then individually measured from crown to extended toe. The data thus obtained were averaged and standard deviations determined, as shown in Tables 1, 2, and 3.

RESULTS

The data in the first experiment (Table 1) show that the average weight of the cortisone injected embryos is less than one-half that of either the saline or sham operated controls; the cortisone group also shows a significantly lower average weight than the cholesterol injected embryos, but the difference is not as great. As to length measurements, the difference between the cortisone and cholesterol groups is not significant, but the saline and sham operated groups each show an average length significantly greater than the other two.

TABLE 2. INJECTIONS ON DAYS 0, 2, 4 AND 6: EXAMINED ON DAY 7

Experiment	Dose	Survival rate	Average weight	Average length
Cortisone Acetate	1 mg. in 2 ml.	27/40	0.65 ± .04* gm. (.65– .78)**	15.5 ± 0.5* mm. (15.0–16.5)**
Sodium Desoxycholate	1 mg. in 2 ml.	21/26	1.41 ± .10 gm. (1.25–1.60)	22.6 ± 0.7 mm. (21.5–23.5)
Sodium Chloride	180 mg. in 2 ml.	14/20	1.56 ± .05 gm. (1.50–1.65)	19.9 ± 0.9 mm. (18.0–21.5)
Sham Operated		36/40	1.27 ± .05 gm. (1.20–1.35)	21.1 ± 0.5 mm. (20.5–22.0)

* Standard deviation.
** Range of variation.

The weight data in the second experiment (Table 2) also show that the average weight of the cortisone injected embryos is less than one-half that of either the desoxycholate or saline injected controls, and only slightly more than one-half that of the sham operated group. While the data on length measurements are not as striking, they also show that, on the average, cortisone injected embryos are significantly shorter than embryos in any of the three control groups.

Whereas, in the first two experiments the growth retardation in the cortisone injected embryos is more strikingly manifested in changes in body weight than in body length, in the third experiment the opposite appears to be the case. While, on the average, the weight is lower in the cortisone injected embryos than in the three control groups, the difference between the embryos receiving cortisone and those receiving sodium desoxycholate or saline is not significant. On the other hand, the average length is significantly less in the cortisone injected embryos than in the three control groups. The fact that sodium desoxycholate and sodium chloride produce some growth retardation, as manifested by both weight and length measurements, suggests that the mere introduction of fluid into very young chick embryos may have some effect in slowing growth processes.

TABLE 3. INJECTED ON DAYS 0 AND 2: EXAMINED ON DAY 4

Experiment	Dose	Survival rate	Average weight	Average length
Cortisone Acetate	1 mg. in 0.2 ml.	29/39	.023 ± .002* gm. (.021–.025)**	.087 ± .002* mm. (.082–.090)**
Sodium Desoxycholate	1 mg. in 0.2 ml.	27/39	.031 ± .006 gm. (.020– .040)	.100 ± .009 mm. (.085–1.250)
Sodium Chloride	180 mg. in 0.2 ml.	18/39	.030 ± .006 gm. (.022– .038)	.108 ± .008 mm. (.100– .125)
Sham Operated		20/39	.038 ± .004 gm. (.032– .040)	.126 ± .005 mm. (.115– .135)

* Standard deviation.
** Range of variation.

DISCUSSION

These results show that when cortisone is administered on the initial day of incubation and at 2 or 3 day intervals thereafter, there is a retardation of growth which can be detected as early as the fourth day of incubation, and is also demonstrable on the seventh and tenth incubation days. The use of non-hormonal steroids as one of the controls serves to indicate that this growth retardation is, in all likelihood, a specific hormonal effect. Furthermore, the data show that, contrary to the report of Karnofsky *et al.* (6), this effect by cortisone can be demonstrated before the eighth incubation day. This growth retardation is more strikingly manifested in the alteration of the average length of the embryo in the earliest period and in the alteration in the average weight in the later periods. Although these experiments do not provide an explanation of this difference, it may be related to the fact that during the earliest periods of embryogenesis processes of differentiation are predominant, while at later periods processes of growth become more pronounced. The weight differences observed in these experiments cannot be accounted for on the basis of retention of tissue fluids, since the well-known property of adrenal corticoids of causing a retention of water and electrolytes would tend to increase, rather than to decrease, the weight of embryos receiving cortisone.

As has been pointed out, the suppression of protein synthesis by cortisone is believed by Allbright (1) to be an "anti-anabolic" effect, the mechanism of which Li (8) would apparently consider to be that of an antagonism to hypophyseal Growth Hormone. On the other hand, the observation of Buno and Goyena (2) that cortisone can cause an *in vitro* inhibition of growth of chick embryo femur may be taken to indicate that the latter hormone is also capable of inhibiting growth which may not be somatotropin dependent. The present observations appear further to support such a conclusion, since it is unlikely that hypophyseal Growth Hormone is active as early as the fourth day of embryogenesis of the chick. Rahn (9)

has reported that Rathke's pouch becomes metamorphosed into a circumscribed mass of solid cell strands within a mesenchymatous sheath by about the fifth day. The entire buccal portion of the hypophysis shows an affinity for basic dyes on the eighth day, but the basophilic and acidophilic cells do not differentiate as such until the tenth day. It would thus appear unlikely that somatotropin secretion occurs before the tenth day, and thus all three of our experiments may be interpreted as demonstrating a growth retardation not dependent upon somatotropin suppression, possibly even a direct inhibition of protein synthesis and growth by cortisone.

Nevertheless, some caution as regards the latter conclusion is in order. Rahn and Drager (10) have pointed out that the time at which hormones are produced by the hypophysis does not necessarily correspond to the time at which staining differences appear; the melanophore-dispersing hormone of the hypophysis is first detected on the fifth day, although definite cellular types do not appear in the gland until the tenth day. However the observation of Fugo (5) that hypophysectomy of the chick embryo has no appreciable effect on its growth and development until after the tenth day of incubation makes it unlikely that endogenous somatoropin is secreted in significant amounts prior to that time. Nevertheless, it is, as yet, not possible to exclude the presence in the yolk of some growth stimulant of maternal origin to which cortisone may be antagonistic. Thus, while the present data indicate that the growth retarding effect of cortisone during the first 10 days of embryogenesis is, in all likelihood, not a manifestation of endogenous somatotropin inhibition, further investigation is necessary to determine whether or not this is a direct effect of cortisone.

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STUDIES OF THE SUPPRESSION OF AMINONITRILE LESIONS IN RATS BY THYROXINE ANALOGUES[1]

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ABSTRACT

In contrast with the striking suppressing effects of triiodothyronine and thyroxine on the lesions produced by aminoacetonitrile in rats, the lesions produced by β-aminopropionitrile were only slightly suppressed by these hormones.

The activity of eight thyroxine analogues in the suppression of the aminoacetonitrile lesions in rats was found to be far below that of triiodothyronine.

Corticosterone and cortisone suppressed only slightly the lesions produced by aminoacetonitrile in rats.

AMINONITRILES administered to vertebrates during a certain period of their growth cause extensive derangement of the mesodermal tissues, including skeletal deformities (1, 2, 3). Of the many aminonitriles tested we have found only three which produce the mesodermal lesions: aminoacetonitrile (AAN) (4), methyleneaminoacetonitrile (MAAN) (5), and β-aminopropionitrile (BAPN) (4). The last compound is found in the form of β-(γ-L-glutamylamino) propionitrile in the seeds of certain legumes of the Lathyrus family (6). The lesions produced by these three aminonitriles appear histologically to be similar, although in the rat AAN and MAAN are about four times more active than BAPN (5). However, certain differences have been observed in the action of these compounds. For example, frog larvae are responsive to very low concentrations of BAPN but are not affected by AAN (3). Dissecting aneurysm of the aorta is frequently observed in weanling rats fed BAPN but rarely in rats fed AAN or MAAN. The epiphyseal plates of rats treated with AAN or MAAN are wider but have fewer rents than the plates of rats fed BAPN. Kyphoscoliosis develops very frequently in rats treated with BAPN and rarely in rats treated with AAN. Dasler has found that semicarbazide and mercaptoethylamine produce lesions in rats similar to the BAPN lesions (7, 8).

Lately we have described suppression of the lesions produced by AAN with low doses of L-3:5:3′-triiodothyronine (TRI) (9); the first series of experiments reported here has shown that TRI has little suppressing effect upon the lesions produced by BAPN.

Triiodothyropropionic acid and tetraiodothyropropionic acid have been

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found to be much more active than TRI in accelerating the metamorphosis of tadpoles of *Rana esculenta* (10). In the second series of experiments here reported, none of a number of analogues of TRI and thyroxine tested was found to be as active as TRI in the suppression of the AAN lesions.

Selye and Bois (11) have described the suppressing effect of cortisol acetate upon the lesions produced by AAN in rats. Our results with the administration of corticosterone and cortisone acetate[2] in rats treated with aminoacetonitrile are here reported.

I. *Effect of Thyroxine and Triiodothyronine on the Lesions Produced by β-aminopropionitrile in Rats*

In the first experiment (Table 1) weanling male Sprague-Dawley rats weighing 42 to 50 gm. were fed *ad libitum* ground Purina chow containing

TABLE 1. EFFECT OF L-THYROXINE (TX), L-TRIIODOTHYRONINE (TRI) AND ANALOGUES IN PREVENTION OF SKELETAL LESIONS IN RATS GIVEN PURINA CHOW CONTAINING 0.18% OF β-AMINOPROPIONITRILE[1] (BAPN)

Treatment[2] (μg./day/rat)	Average		Severity of lesions
	Starting weight (gm.)	Final weight (gm.)	
Purina only[3]	49	157	0
Purina + BAPN[3]	49	126	3+
Tx			
50	48	129	3+
125	48	118	2+
TRI			
2[4]	49	127	3+
9[5]	49	97	2+
3:5:3′:5′-Tetraiodothyropropionic acid			
2	48	124	3+
9	48	122	3+
3:5:3′-Triiodothyropropionic acid			
2	48	123	3+
9	44	120	3+

5 rats were used to test each dose. Duration of experiment, 18 days.

[1] Given as salt of fumerate.

[2] Tx, TRI and analogues were administered in slightly alkaline solutions and injected subcutaneously.

[3] 0.2 ml. normal saline injected daily.

[4] One rat died on the 17th day of the experiment of dissecting aneurysm of the aorta.

[5] Two rats died on the 17th day of experiment: one had a dissecting aneurysm of the aorta; no cause of death was found for the other.

0.18% β-aminopropionitrile fumarate.[3] The fumarate salt of the BAPN is a solid and easier to use in feeding experiments than the liquid BAPN. The animals were housed in cages in groups of five. Various doses of thyroxine (Tx), TRI, 3:5:3′:5′-tetraiodothyropropionic acid, and 3:5:3′-triiodothyropropionic acid were injected subcutaneously daily in slightly alkaline solutions. Two groups of rats served as controls: one group was fed

[2] Cortone, Merck & Co.

[3] Courtesy of Abbott's Laboratories, Chicago, Illinois.

Purina chow alone; another group received the BAPN diet alone. The rats in the control groups received 0.2 ml. normal saline injected daily. The animals were weighed every four days. Duration of the experiment was 18 days.

One rat of the group receiving BAPN diet plus TRI (2 μg. daily) died of dissecting aneurysm of the aorta on the seventeenth day of the experiment. On the same day two rats died of the group receiving BAPN diet plus TRI (9 μg. daily). At the post mortem examination one rat had a massive hemothorax due to a dissecting aneurysm of the aorta; no cause of death was found for the other animal.

In the second experiment (Table 2) the rats were fed for 18 days Purina

TABLE 2. EFFECT OF L-THYROXINE (Tx) AND L-TRIIODOTHYRONINE (TRI) IN PREVENTION OF SKELETAL LESIONS IN RATS GIVEN PURINA CHOW CONTAINING LATHYRUS ODORATUS MEAL (35% FOR FIRST 18 DAYS AND 10% FOR FOLLOWING 12 DAYS)

Treatment (μg./day/rat)	Average		Severity of lesions
	Starting weight (gm.)	Final weight (gm.)	
(A) Purina only[1]	55	227	0
Tx 50	54	230	0
Tx 100	54	245	0
TRI 2	54	233	0
TRI 6	54	239	0
TRI 9	54	192	0
(B) Purina+*L. odaratus*[1,2]	55	136	4+
Tx 50	54	181	3+
Tx 100[3]	54	147	2+
TRI 2[4]	54	178	3+
TRI 6[5]	54	162	3+
TRI 9[6]	54	141	2+

5 rats were used to test each dose in the experimental and in the control groups. Duration of experiment, 30 days.

[1] 0.2 ml. normal saline injected daily.

[2] One rat died on the 14th day of the experiment of dissecting aneurysm of the aorta.

[3] Two rats died on the 13th day of the experiment, cause undetermined. One rat died on the 21st day of the experiment of dissecting aneurysm of the aorta.

[4] One rat died on the 22nd day of the experiment of dissecting aneurysm of the aorta.

[5] Two rats died on the 14th day of the experiment, cause undetermined.

[6] Two rats died of dissecting aneurysm of the aorta, one on the 20th and the other on the 24th day of the experiment.

chow containing 35% finely ground *Lathyrus odoratus* meal. Mild skeletal lesions were observed in the roentgenograms taken on the 18th day of the experiment. A diet containing 50% *Lathyrus odoratus* meal was then administered for the following 12 days. Various doses of Tx and TRI were injected daily in slightly alkaline solutions. Five groups of rats fed Purina chow alone receiving similar doses of Tx and TRI served as controls. The rats of two other control groups were injected daily with 0.2 ml. normal saline. One of these groups was fed Purina chow alone and the other the *Lathyrus odoratus* diet. Duration of the experiment was 30 days. One rat receiving the *Lathyrus odoratus* diet and normal saline died of dissecting

aneurysm of the aorta on the 14th day of the experiment. Four rats of the experimental groups receiving Tx or TRI died of dissecting aneurysm of the aorta between the 20th and 24th days of the experiment. Four rats of the same groups died between the 13th and 14th days of the experiment; the post mortem examination did not reveal any abnormality to explain the cause of death in these rats.

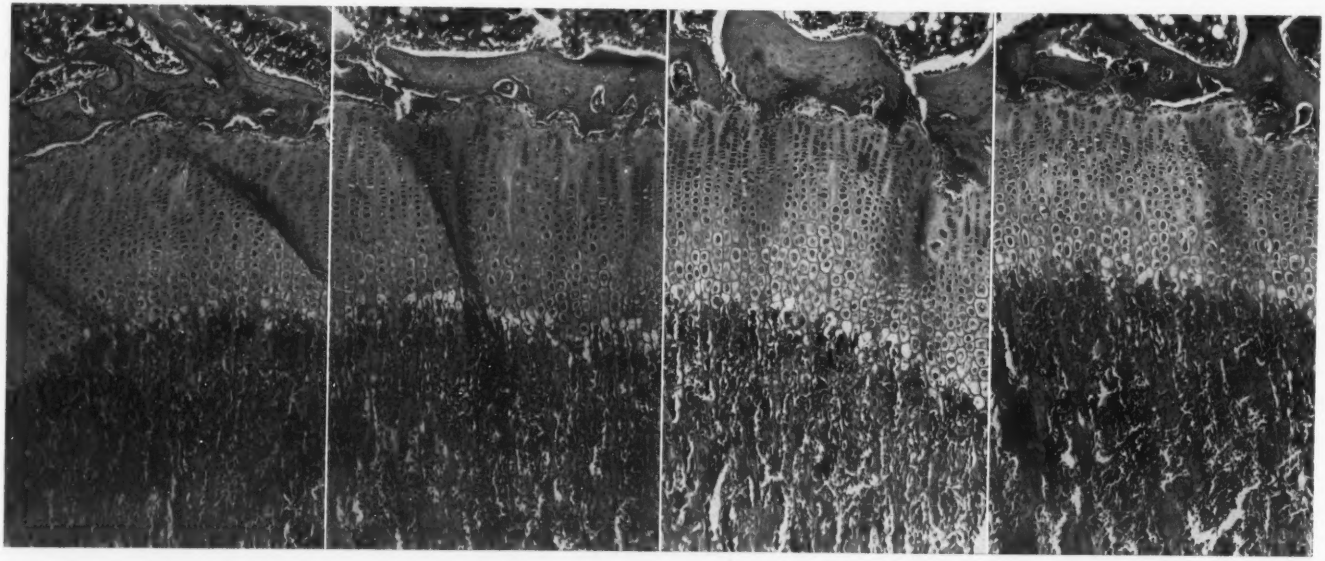
At termination of each experiment the animals were sacrified with ether and roentgenograms were made. At the post mortem examination the knees, including lower half of femur and upper half of tibia, were excised and after fixation in 10 per cent neutralized formalin were decalcified in 6 per cent aqueous solution of sulfosalicylic acid. Sagittal sections were stained with hematoxylin and eosin. The roentgenograms were evaluated for a) width of the epiphyseal plates adjoining the knee, b) amount of periosteal new bone formation, c) severity of the kyphoscoliosis, d) width of the ilium, and e) severity of the osteoporosis. The approximate estimate of severity of the lesions obtained from the roentgenograms was checked against the lesions observed in histological sections. Rats of the same experimental group showed only slight variations in the severity of the skeletal lesions.

In contrast with the striking suppressing effects of Tx and TRI on the skeletal lesions produced by AAN (9), the lesions produced by BAPN were only slightly suppressed by the thyroid hormones. The animals on the highest doses of Tx (125 μg./day) and TRI (9 μg./day) had the least severe lesions. The rats of the control groups receiving TRI (9 μg./day) had epiphyseal plates narrower than the normal animals with fewer cartilage cells, indicating retardation of growth (Fig. 1). However, the epiphyseal plates of the experimental animals on a similar dose of TRI were wider than those of normal animals and rents were apparent in the proliferating zone of the plate. The abnormalities were even more pronounced in rats on the *Lathyrus odoratus* diet which received 2 μg./day of TRI or 100 μg./day of Tx (Fig. 2). The kyphoscoliosis was less severe in the animals receiving TRI 9 μg./day but was not completely prevented, neither were osteoporosis, lesions of the aorta, and bone deformities.

II. *Relative Potencies of Tx and TRI Analogues in the Suppression of AAN Lesions in Rats*

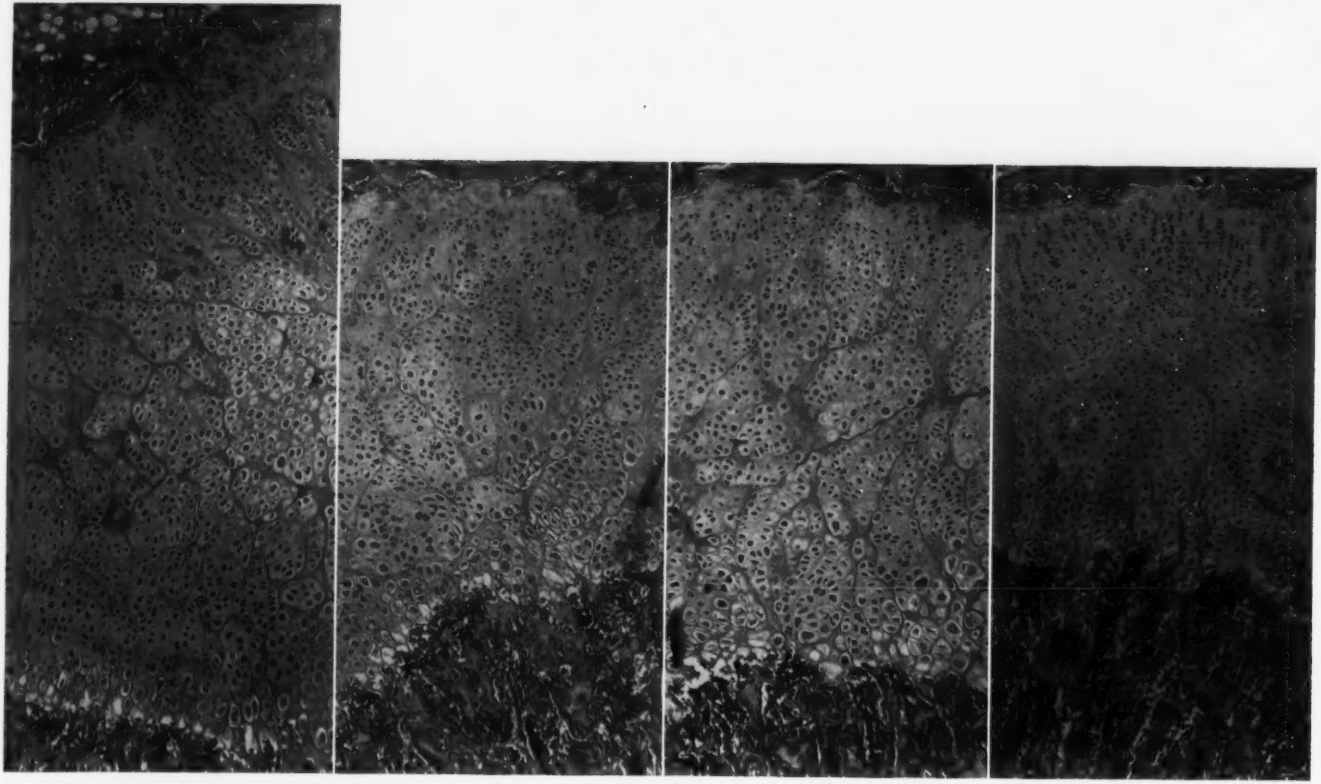
In our quest for a compound which, when administered in non-toxic doses, would arrest the lesions produced by the aminonitriles, we tested a variety of Tx and TRI analogues (Table 3). Groups of six weanling male Sprague-Dawley rats fed Purina chow were injected subcutaneously daily with 6 mg. AAN for the first 8 days of the experiment and 9 mg. AAN for the following 9 days. Tx, TRI, or the analogues were injected during this period in the doses indicated in Table 3.[4] Two groups of rats served as controls: One group was fed Purina chow alone, another group received the AAN injections without thyroid hormones or analogues. Duration of the experiment was 17 days. At the termination of the experiment the animals were sacrified and roentgenograms were

[4] The author is indebted to Dr. Robert L. Kroc of the Warner Chilcott Laboratories for kindly providing the analogues used in this experiment.



A B C D

FIG. 1. Proximal epiphyseal plates of the tibia of 56-day-old rats (×40, hematoxylin and eosin stain) treated as follows for 30 days:
A. Normal control.
B. Tx (100 μg. per day) injected subcutaneously.
C. TRI (2 μg. per day) injected subcutaneously.
D. TRI (9 μg. per day) injected subcutaneously.



A B C D

FIG. 2. Proximal epiphyseal plates of the tibia of 56-day-old rats (×40, hematoxylin and eosin stain) treated as follows for 30 days:
A. *Lathyrus odoratus* diet (35% for the first 18 days and 50% for the following 12 days).
B. *L. odoratus* diet plus Tx (100 μg. per day) injected subcutaneously.
C. *L. odoratus* diet plus TRI (2 μg. per day) injected subcutaneously.
D. *L. odoratus* diet plus TRI (9 μg. per day) injected subcutaneously.

made. At postmortem examination no instance of dissecting aneurysm of the aorta was noted in either control or experimental animals. The knees were excised and processed for histological studies as in the previous experiments. An approximate estimate of the severity of lesions obtained from the roentgenograms was checked against the lesions observed in the histological sections.

As in previously reported experiments (9), TRI was estimated to be at least 50 times more active than Tx in the suppression of the lesions pro-

TABLE 3. EFFECT OF INJECTED L-TRIIODOTHYRONINE (TRI), THYROXINE (Tx), AND ANALOGUES IN PREVENTION OF SKELETAL LESIONS IN RATS TREATED WITH 6 MG./DAY OF AMINOACETONITRILE (AAN) FOR 8 DAYS AND 9 MG./DAY AAN FOR FOLLOWING 9 DAYS ADMINISTERED SUBCUTANEOUSLY

Treatment) (g./day/rat)		Average		Severity of lesions
		Starting weight (gram)	Final weight (gram)	
Purina only[1]		61	162	0
Purina + AAN		68	87	4+
Tx 10		68	113	4+
Tx 50		67	133	4+
Tx 100		67	142	1+
TRI 1		67	140	1+
TRI 2		65	106	)
TRI 10		65	90	)
3:5:3′-Triiodothyroacetic acid	10	66	132	1+
	100	66	113	1+
3:5:3′-Triiodothyropropionic acid	10	65	128	4+
	100	65	130	2+
3:5:3′:5′-Tetraiodothyroacetic acid	10	64	112	4+
	100	65	132	1+
3:5:3′:5′-Tetraiodothyropropionic acid	10	63	92	4+
	100	65	135	2+
L-3:3′:5′-Triiodothyronine	10	66	121	4+
	100	66	115	3+
3:3′:5′-Triiodothyropropionic acid	10	65	125	4+
	100	60	136	3+
D,L-3:3′-Diiodothyronine	10	68	120	4+
	100	68	127	3+
3:3′-Diiodothyropropionic acid	10	66	136	4+
	100	63	98	3+

6 rats were used to test each dose. Purina chow was fed *ad lib.*
[1] 0.2 ml. normal saline injected daily.

duced by AAN. The activity of the analogues tested was far below that of TRI. The compounds 3:5:3′-triiodothyroacetic acid, 3:5:3′-triiodothyropropionic acid, 3:5:3′:5′-tetraiodothyroacetic acid and 3:5:3′5′-tetraiodothyropropionic acid were found to have an activity similar or slightly below that of Tx. The compounds with the iodine atoms in position 3:3′:5′ in the thyronine molecule showed practically no activity in our experiments. Neither 3:3′-diiodothyronine nor the propionic acid analogue showed any significant activity.

An attempt was made to use the suppressing effect of thyroid hormones and analogues on the AAN lesions in weanling rats as a "bioassay" for these compounds. For this purpose all the analogues listed in Table 3 were administered also in doses of 2 μg. and

TABLE 4. APPROXIMATE RELATIVE POTENCIES OF THYROXINE AND TRIIODOTHYRONINE ANALOGUES IN THE SUPPRESSION OF AMINOACETONITRILE LESIONS IN RATS

Thyroxine	100
L-3:5:3′-Triodothyronine	5000
3:5:3′-Triiodothyroacetic acid	100
3:5:3′-Triiodothyropropionic acid	90
3:5:3′:5′-Tetraiodothyroacetic acid	90
3:5:3′:5′-Tetraiodothyropropionic acid	75
L-3:3′:5′-Triiodothyronine	0
3:3′:5′-Triiodothyropropionic acid	0
D,L-3:3′-Diiodothyronine	0
3:3′-Diiodothyropropionic acid	0

50 μg. per day to groups of six rats receiving the AAN treatment. At the end of the experiments an estimate was made of the severity of the skeletal lesions as observed in the roentgenograms and in the histological sections. Although the rows of epiphyseal cartilage cells were distorted in many animals it was possible to make an approximate cell count of several rows and obtain the average number of cells per row. A similar area of the upper tibial epiphyseal plate was counted in each animal. The cell count was checked against the dose of thyroid hormone or analogues received by the animal. Table 4 shows the approximate relative potencies of the compounds tested.

III. *Effect of Corticoids on the AAN Lesions in Rats*

The work of Selye and collaborators (11) on lathyrism stimulated us to test corticosterone and cortisone. Groups of five weanling male Sprague-Dawley rats fed Purina chow were injected daily subcutaneously with 7.5 mg. AAN for the first 18 days of the experiment and 12 mg. AAN for the following 6 days. The daily doses of corticosterone or cortisone indicated in Table 5 were simultaneously injected. Estrone (20 μg. per day) was also tested. Four groups of rats served as controls. One control group received the AAN injections and not the corticoids. The other three control groups did not receive AAN: normal saline (0.2 ml. per day) was injected to the rats of one group, corticosterone

TABLE 5. EFFECT OF INJECTED CORTICOSTERONE, CORTISONE (3) AND ESTRONE IN PREVENTION OF SKELETAL LESIONS IN RATS TREATED WITH 7.5 MG./DAY AMINOACETONITRILE (AAN) FOR 18 DAYS AND 12 MG./DAY FOR FOLLOWING 6 DAYS ADMINISTERED SUBCUTANEOUSLY

Treatment Daily dose/rat		Average		Severity of lesions
		Starting weight (gm.)	Final weight (gm.)	
A) Purina only[1]		64	180	0
Corticosterone[2]	2 mg.	67	195	0
Cortisone[4]	2 mg.	63	97	0
B) Purina + AAN[1]		63	156	4+
Corticosterone	1 mg.	64	178	3+
Corticosterone	2 mg.	67	162	2+
Cortisone[3]	1 mg.	68	129	3+
Cortisone	2 mg.	63	98	2+
Estrone	20 μg.	67	154	4+

5 rats were used to test each dose in the experimental and in the control groups. Duration of experiment, 24 days.

[1] 0.2 ml. normal saline injected daily.

[2] One rat died on the 18th day of the experiment of dissecting aneurysm of the aorta.

[3] Upjohn Company.

[4] Cortone, Merck & Co.

(2 mg. per day) was injected to the rats of another group, and cortisone (2 mg. per day) was injected to the rats of the last group. One rat of the group receiving AAN and 1 mg. per day of the cortisone died of dissecting aneurysm of the aorta on the eighteenth day of the experiment. At the termination of the experiment the animals were sacrificed and roentgenograms were made. The knees were excised and processed for histological studies as in the previous experiments.

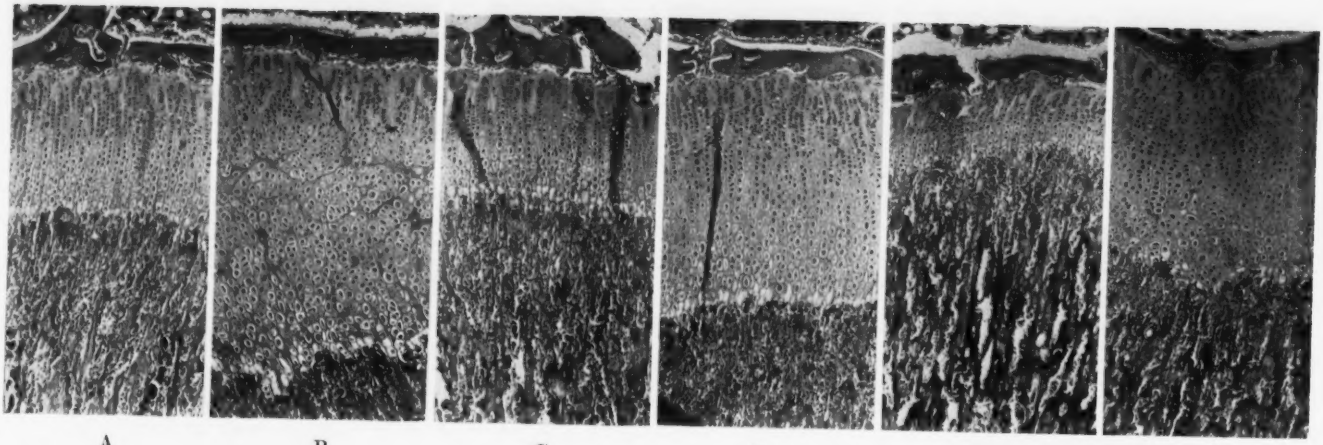
The experimental animals which had received corticosterone had skeletal lesions slightly less severe than the animals on AAN alone. The epiphyseal plates of the control rats which had been given corticosterone were somewhat narrower than those of the normal animals. Cortisone markedly slowed the growth of the rats but did not suppress completely the AAN lesions. The epiphyseal plates of the control animals on cortisone were very narrow and the endochondral ossification was greatly deranged (Fig. 3). Estrone was observed to have no suppressing effect on the skeletal lesions produced by AAN.

DISCUSSION

The pathogenesis of the lesions induced in experimental animals by the administration of aminonitriles is not fully understood. The aminonitriles affect a definitely limited stage of the process of mesodermal tissue differentiation, and they do not seem to cause any breakdown of completed mesodermal structures (12). Recent studies indicate that aminonitriles may impair collagen and elastin formation in wound healing (13), in croton oil pouches (14), and in the aorta (12, 15). The epiphyseal plate, being a remnant of continually maturing embryonal tissue, is particularly vulnerable to the action of the aminonitriles (2).

Castellani and Castellani (16) have observed a significant decrease of hexosamine content in epiphyseal plates of young rabbits treated with AAN as compared with the normal; this suggests a decrease in the content of mucopolysaccharides. The same authors have found the hydroxyproline (hence total collagen) content of epiphyseal plates from rabbits treated with AAN not reduced from the normal. This observation has been corroborated in rats by Follis and Tousimis (17) who at the same time have detected with the electron microscope a great reduction of collagen fibrils in the epiphyseal cartilage homogenates of AAN-treated rats. It is suggested that the aminonitriles may cause a failure of the tropocollagen molecule to form collagen fibers. Perhaps the defective mucopolysaccharides of the epiphyseal cartilage are responsible for this failure.

TRI had by far the greatest suppressing effect upon the AAN lesions of all the hormones tested. Tx in doses 50 times greater had a similar effect. However, TRI and Tx caused only a slight suppression of the lesions caused by BAPN. This suggests that there may be a difference in the nature of the biochemical derangement caused by these two aminonitriles although histologically the lesions appear similar. Thus a new approach be-



A B C D E F

FIG. 3. Proximal epiphyseal plates of the tibia of 55-day-old rats (×30, hematoxylin and eosin stain) treated as follows for 24 days:
A. Normal control.
B. AAN (7.5 mg. per day) for 18 days and AAN (12 mg. per day) for the following 6 days administered subcutaneously.
C. Corticosterone (2 mg. per day) administered subcutaneously.
D. AAN as in B plus corticosterone (2 mg. per day) administered subcutaneously.
E. Cortisone (2 mg. per day) administered subcutaneously.
F. AAN as in B plus cortisone (2 mg. per day) administered subcutaneously.

comes available for study of the action of thyroid hormones upon connective tissues. The suppression of the AAN lesions by Tx and TRI may be related to the effects of these hormones in promoting maturation (18, 19). No explanation can be offered at this time why there is only a very slight suppressing effect of these hormones on the lesions produced by BAPN.

The activity of Tx and TRI analogues tested in our experiments corresponded closely with the activity of these compounds in the suppression of the thiouracil-induced goiter in rats (10). Roche *et al.* (10) reported that 3:5:3′-triiodothyropropionic acid was 290 times more active than Tx in its effect on the metamorphosis of tadpoles. The activity of these two compounds was found to be almost equal in the suppression of the AAN lesions in rats.

Selye and Bois (11) have described the suppression with cortisol acetate of the skeletal lesions produced by AAN in rats with one kidney removed and given a 1% NaCl solution as drinking fluid. In our experiments with intact rats neither corticosterone nor cortisone suppressed the AAN lesions completely, although these were less severe than in control animals receiving AAN alone. Inhibition of bone growth and structural changes in the metaphysis produced by cortisone have been previously described (20, 21, 22). We observed great narrowing of the epiphyseal plates and the derangement of the endochondral ossification in the control rats injected with cortisone. However, the AAN-cortisone treated rats had epiphyseal plates wider than the normal animals, with disarranged cartilage cell columns. Corticosterone was found to be less effective than cortisone in the control of the epiphyseal plate lesions. Testosterone failed in previous experiments (2) to arrest the lesions produced in rats with *Lathyrus odoratus* meal. Estrone was found likewise to be ineffective in checking the lesions produced by AAN in the experiments here reported.

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THE EFFECT OF ADRENAL AND PITUITARY HORMONES ON THE URINARY EXCRETION OF IODIDE IN MICE[1]

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ABSTRACT

The urinary excretion of radioiodide was measured in mice injected with a variety of adrenal and pituitary hormones.

While radioiodine excretion was diminished in adrenalectomized mice and restored to a nearly normal rate by cortisone, this hormone, as well as aldosterone, deoxycorticosterone, adrenaline and noradrenaline, had little effect in intact mice. ACTH was slightly ioduretic and TSH had no effect.

A considerable increase in radioiodide excretion was observed after acute injection of the posterior pituitary hormones, vasopressin and oxytocin. The effect of vasopressin did not depend on the amount of impurity present in the preparations tested, and varied linearly with the logarithm of the dose. This hormone increased the renal clearance of radioiodide from blood. These observations suggest that the posterior pituitary plays a role in the control of iodide excretion.

When vasopressin was administered for prolonged periods of time, the resulting iodide losses, unlike those produced by sodium chloride administration, did not induce a stimulation of the thyroid gland. In fact, vasopressin depressed the thyroid gland since the thyroid weight was reduced to about 40% of that in controls. Presumably, this effect prevented the iodine losses from resulting in thyroid stimulation.

THE urinary excretion of iodide in mice is increased 10 to 20 times by a single dose of sodium chloride (1). When this salt is administered over a long period of time, the iodide losses induce a relative iodine deficiency which may result in thyroid stimulation and even goiter (1, 2).

This work was followed up by investigating the influence of various factors on ioduria. When a number of salts other than sodium chloride were tested, the results obtained led to the conclusion that ioduresis is induced by an increase in the ionic concentration of blood, no matter which ions produce this increase (3). The effect of hormonal factors on ioduria was

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examined in the experiments now to be reported. The posterior pituitary hormone vasopressin was found to increase the renal excretion of iodide considerably. It was therefore investigated whether or not prolonged treatment with this hormone can result in thyroid stimulation and goiter, as in the case of treatment with sodium chloride.

MATERIAL AND METHOD

The hormones were tested in 2 to 9-month old C_3H and albino mice. Males were used in all experiments except one (ascorbic acid, Table 1). Since sodium chloride was known to increase the urinary excretion of iodine (1), the mice were fed for 3 weeks prior to the hormone administration on a diet containing only traces of sodium chloride (2). This diet, which contained less than 3 μg. iodine per 100 gm., consisted of 89% corn meal, 10% dried brewer's yeast and 1% calcium carbonate. However, two of the experiments (reported in Fig. 1 and Table 3) were carried out in mice fed Purina fox chow.

In all acute experiments, each mouse received simultaneously 2 subcutaneous injections of 0.2 ml. aqueous solutions containing respectively the hormone and 10 μc. carrier free I^{131}. The animal was then placed in a 250 ml. beaker fitted with a wire mesh floor allowing the urine to settle at the bottom and was given access to distilled water but received no food. Two and a half hours later, the mouse was anaesthetized with ether in the beaker, and the thyroid gland was removed. The I^{131} content of urine (including that in the bladder) and of the thyroid was then measured as described previously (1).

The effect of sodium chloride on the renal clearance of radioiodine from the blood was investigated by measuring the radioiodine content of urine and blood 1 and 3 hours after I^{131} administration as described earlier (1).

The following hormones were used: *vasopressin:* Pitressin from Parke, Davis (55 U.S.P. units per mg.); in one experiment (Table 3) two preparations (200 and 400 U.S.P. units per mg.) obtained from Dr. B. G. Benfey, Department of Pharmacology, McGill University, were also tested. *Vasopressin tannate* in peanut oil: Parke, Davis. *Oxytocin:* Dr. B. G. Benfey. *Thyrotrophic hormone* (TSH): Nordic Biochemicals, Montreal. *Adrenocorticotrophic hormone* (ACTH): Armour. *Aldosterone:* Dr. I. Dyrenfurth, Department of Investigative Medicine, McGill University. *Deoxycorticosterone acetate:* Nutritional Biochemicals Corp. L-*adrenaline:* Eastman. D,L-*noradrenaline:* Dr. A. S. V. Burgen, Department of Physiology, McGill University. *Cortisone acetate:* Merck.

RESULTS

Adrenal hormones (Table 1)

Urinary excretion and thyroid uptake of radioiodine were not significantly influenced by cortisone, but were significantly decreased by adrenalectomy (performed immediately before treatment) while the adminstration of cortisone to adrenalectomized animals restored a normal condition.

The other adrenal hormones were tested in intact animals. They did not influence urinary excretion, except for adrenaline which decreased it.

The thyroid uptake of radioiodine was decreased by adrenaline and noradrenaline[2] and slightly increased by aldosterone. No effect of ascorbic acid was observed.

[2] The effect of adrenaline and noradrenaline was reported in detail elsewhere (4).

Pituitary hormones

Adrenocorticotrophic hormone and two posterior pituitary hormones, vasopressin and oxytocin, significantly increased the urinary excretion of radioiodine, while thyrotrophic hormone had no effect (Table 2). None of these hormones modified the thyroid uptake of radioiodine in the experiment reported in Table 2, but in other experiments vasopressin significantly decreased it (4).

TABLE 1. THE ACUTE EFFECT OF ADRENAL HORMONES ON THE URINARY EXCRETION AND THYROID UPTAKE OF RADIOIODINE

Treatment	No. of mice*	% of injected radioiodine in	
		Urine Mean ±S.E.	Thyroid Mean ±S.E.
Sham adrenalectomy, Controls	6	10.1±2.8	11.9±1.9
Sham adrenalectomy, 2 mg. cortisone acetate	6	7.8±1.8	11.6±1.5
Adrenalectomy†	6	*3.2±1.0*	*7.0±1.1*
Adrenalectomy†, 2 mg. cortisone acetate	6	7.0±2.4	13.3±2.5
Controls	8	2.4±0.6	11.7±2.0
1 mg. deoxycorticosterone acetate‡	7	1.1±0.4	15.2±2.4
Controls	8	5.1±0.7	19.7±2.1
1 μg. aldosterone	8	3.3±0.6	*27.6±2.2*
Controls	8	4.0±0.5	20.5±1.2
1 μg. adrenaline	8	2.6±0.5	*16.3±1.2*
50 μg. adrenaline	8	*1.4±0.2*	*5.7±0.7*
0.9 μg. noradrenaline	8	4.7±1.0	16.8±1.8
46 μg. noradrenaline	8	4.6±0.8	*11.6±1.1*
Controls	8	3.1±0.6	10.4±3.6
1 mg. ascorbic acid	8	2.6±0.4	10.6±5.8

* C_3H mice aged 4, 9, 9, 5 and 9 months respectively from the first to the fifth experiment.

† Adrenals removed immediately prior to treatment.

‡ Dissolved in polyethyleneglycol. Controls received polyethyleneglycol.

The italicized figures are significantly different from those of the respective controls.

The effect of vasopressin was further investigated by injecting 1 U.S.P. unit of 3 different preparations of the hormone. In spite of differences in the purity of the three preparations, they increased the urinary excretion of radioiodine to the same extent (Table 3). Thus it was concluded that the active principle was not an impurity but pitressin itself.

When graded doses of vasopressin were injected to Purina fed mice, the amount of radioiodine excreted in the urine varied linearly with the logarithm of the dose (Fig. 1), the increment being 9% of the amount of radioiodine injected for a 10-fold increase in vasopressin dose.

The effect of vasopressin on the renal clearance of radioiodine from the blood was investigated by measuring the urine content and the blood level of this isotope at 1 and 3 hours following administration of the hormone. During the two-hour period comprised between the first and third hour,

TABLE 2. THE ACUTE EFFECT OF PITUITARY HORMONES ON THE URINARY EXCRETION AND THYROID UPTAKE OF RADIOIODINE

Treatment	Number of mice*	% of injected radioiodine in	
		Urine Mean ±S.E.	Thyroid Mean ±S.E.
Controls	8	5.1 ±0.7	19.7 ±2.1
1 U.S.P. unit TSH	8	7.3 ±0.9	15.1 ±2.7
1 mg. ACTH	7	*11.7 ±2.7*	22.9 ±4.1
1 U.S.P. unit vasopressin	7	*20.2 ±3.1*	16.4 ±1.5
1 U.S.P. unit oxytocin	8	*16.5 ±2.1*	23.0 ±1.4

* C_3H 9-month-old mice.
The italicized figures (urine I^{131}) are significantly different from that of controls.

the mean volume of blood cleared per minute by the kidney was 7 microliters in controls and 27 microliters in vasopressin treated animals (Table 4). Thus, the hormone produced a 3.7-fold increase in the renal clearance of radioiodine.

Prolonged administration of vasopressin

Since vasopressin considerably raised the excretion of iodide and since it had been shown previously (2) that large and prolonged losses of iodide caused by sodium chloride ingestion may lead to thyroid stimulation and goiter production, there was a posslbility that prolonged administration of vasopressin would also induce goitrous thyroids. This possibility was investigated in mice fed the diet described above, and injected subcutaneously with 0.5 unit vasopressin in 0.05 ml. water twice daily for 50 days. Controls were injected 0.05 ml. water at the same rate. After 50 days all animals were given a subcutaneous injection of 10 μc. radioiodine and sacrificed 2.5 hours thereafter. The left thyroid lobe was dissected free of connective tissue, weighed on an analytical balance and homogenized in sodium hydroxide solution for radioactivity counting. The average weight per thyroid lobe was 1.7 ±0.8 in controls and 1.1 ±0.2 in vasopressin treated mice. The respective thyroid uptakes of radioiodine in the two groups were 33.3 ±1.8 and 23.5 ±5.3. Thus, vasopressin did not stimulate the thyroid gland.

TABLE 3. THE ACUTE EFFECT OF 1 U.S.P. UNIT OF VARIOUS VASOPRESSIN PREPARATIONS ON THE URINARY EXCRETION OF RADIOIODINE

Vasopressin preparation	Number of mice*	% of injected radioiodine in	
		Urine Mean ±S.E.	Thyroid Mean ±S.E.
Controls	8	17.1 ±5.7	1.4 ±0.4
Parke Davis (55 U.S.P. units/mg.)	8	*41.0 ±4.1*	2.4 ±0.5
Benfey (200 U.S.P. units/mg.)	8	*43.4 ±4.3*	1.5 ±0.4
Benfey (400 U.S.P. units/mg.)	8	*42.3 ±4.6*	1.2 ±0.3

* C_3H, 5-month-old, Purina fed mice.
The italicized values (urine I^{131}) are significantly different from that of controls.

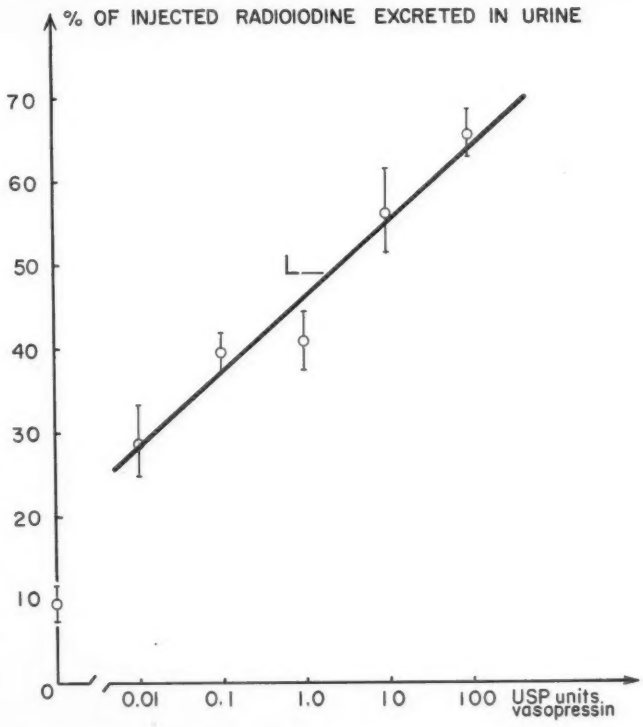

FIG. 1. The acute effect of graded doses of vasopressin on the urinary excretion of radioiodine. The dots indicate the average I^{131} excretion obtained in 7 to 8 albino, 3-month-old, Purina fed mice and the vertical lines the standard errors of the mean. The line L is the best fit line according to the criterion of least squares obtained with the graphic method of Askovitz (14).

The lack of thyroid stimulation after chronic vasopressin administration might be due to the fact that vasopressin eventually became ineffective in augmenting the urinary loss of iodide. However, after one month treat-

TABLE 4. ACUTE EFFECT OF VASOPRESSIN ON BLOOD RADIOIODIDE CLEARANCE BY THE KIDNEY

Treatment	Sacrifice time (hours)	Number of mice*	% of injected I^{131} in		Blood I^{131} renal clearance (ml./minute)
			Urine Mean ±S.E.	1 ml. blood Mean ±S.E.	
Controls	1	7	7.9±2.1	3.8±0.4	0.007
	3	7	11.2±2.8	3.7±0.5	
1 U.S.P. unit vasopressin	1	8	27.9±2.3	2.8±0.3	0.027
	3	8	35.9±4.3	2.3±0.5	

* Albino, 2–3-month-old.

TABLE 5. THE ACUTE EFFECT OF VASOPRESSIN ON THE URINARY EXCRETION OF RADIOIODINE IN MICE PREVIOUSLY ADMINISTERED 1 U.S.P. UNIT VASOPRESSIN DAILY FOR 1 MONTH*

Acute treatment	Number of mice†	% of injected radioiodine in urine Mean ±S.E.
Controls	4	10.7 ±0.6
1 U.S.P. unit vasopressin	4	47.9 ±4.1

* Chronic vasopressin treatment was discontinued during the two days preceding acute vasopressin injection.
† Albino, 2–3-month-old.
Vasopressin significantly increased the urinary excretion of radioiodine.

ment, an acute administration of vasopressin to radioiodine injected mice still elicited the usual ioduresis (Table 5). This hypothesis was therefore rejected.

Another possible explanation for the lack of thyroid stimulation might be the rapid disappearance of vasopressin from the body after injection. A chronic experiment was then carried out as above except for the use of a hormone preparation effective over relatively long periods of time, namely, vasopressin tannate in oil. The effective period of time was determined in a preliminary experiment by injecting 1 U.S.P. unit vasopressin tannate in 0.2 ml. oil and then allowing various periods of time before injecting radioiodine and sacrificing 2.5 hours later. When allowing 4 hours between vasopressin tannate and radioiodine injection, a significant increase in urinary radioiodine was observed. That is the hormone was still effective between 4 and 6.5 hours following injection (Fig. 2). After 8 hours a slight and after 24 hours a very slight effect was observed but these were not significant statistically. After 48 hours definitely no effect was noticeable (Fig. 2). Accordingly, it was decided to administer the preparation once a day. One U.S.P. unit vasopressin tannate in 0.2 ml. oil was injected subcutaneously daily for 30 days to 12 mice. Twelve control mice received a daily dose of 0.2 ml. peanut oil.

TABLE 6. THE EFFECT OF 30 DAYS DAILY ADMINISTRATION OF VASOPRESSIN TANNATE IN OIL ON THE THYROID GLAND

Treatment	Number of mice*		Body Weight (gm.)		Left thyroid lobe weight (mg.) Mean ±S.E.	% of injected radioiodine in thyroid† Mean ±S.E.
	Initial	Final	Initial Mean ±S.E.	Final Mean ±S.E.		
Controls (0.2 ml. oil daily)	12	11	24.6 ±1.0	28.0 ±1.1	0.98 ±0.11	12.2 ±1.8
1 U.S.P. unit vasopressin tanate in 0.2 ml. oil daily	12	5	25.6 ±0.5	*21.2 ±1.1*	*0.38 ±0.06*	9.3 ±0.7

* Albino, 2 months old at the start.
† Left lobe only.

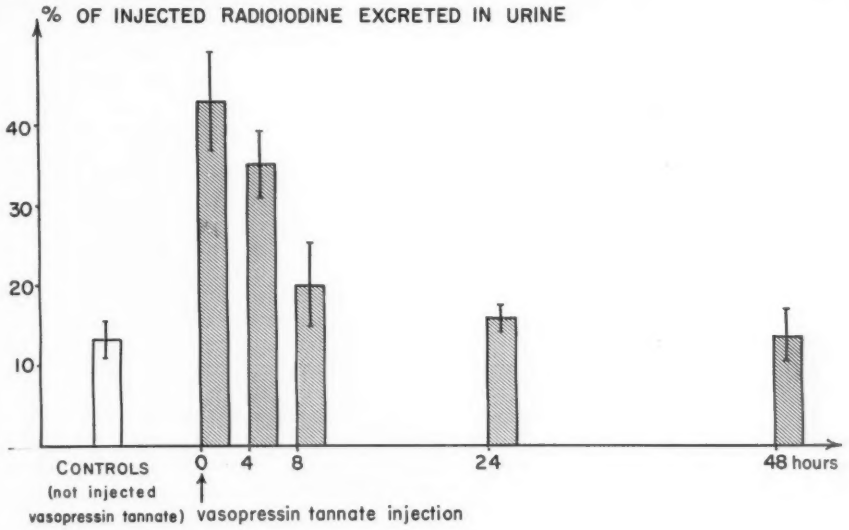

FIG. 2. Duration of effect of 1 U.S.P. unit vasopressin tannate in oil on the urinary excretion of radioiodine. The height of the bars indicates the average I^{131} excretion observed in 7 to 8 albino, 4-month-old mice. The vertical lines are the standard errors. Vasopressin tannate was injected at time 0. (The left side of the bars indicates the time of I^{131} injection and the right side the time of sacrifice, that is, 2.5 hours later.)

While the control mice gained weight and only 1 out of 12 died, the vasopressin-tannate-treated mice lost weight and 7 out of 12 died over the 30-day period. The thyroid weight was significantly lower in vasopressin-tannate-treated mice than in controls. Thyroid uptake of radioiodine was slightly decreased (Table 6). Incidentally, during the course of the experiment, it was noted that the hairs of the vasopressin treated mice were covered with oil, while those of controls were dry. At autopsy large amounts of oil were found subcutaneously in controls, but not in vasopressin treated animals. Perhaps the hormone modified the skin permeability to oil or prevented closure of the holes made by the injection needle.

DISCUSSION

Although adrenal hormones are known to influence the excretion of sodium and other electrolytes (5–8), they had little effect on the excretion of the iodide ion in the intact mouse. The observation that iodide excretion is decreased by adrenalectomy and is restored by cortisone to nearly the same level as in intact mice is in agreement with a similar observation made by Paris *et al.* (9) in the rat and with the fact that cortisone enhances radioiodine excretion in hypophysectomized rats (10). Thus it appears that the presence of intact adrenals is required for an adequate excretion of the

iodide ion. However, exogenous adrenal hormones have little effect in animals with adrenals functioning adequately: cortisone, deoxycorticosterone acetate and aldosterone did not modify iodide excretion. (The moderate decrease in excretion produced by adrenaline is likely to be the result of vasoconstriction.)

In contrast, posterior pituitary hormones considerably enhanced the excretion of iodide in intact animals. The mechanism by which vasopressin produces this effect cannot be explained merely on the basis of alterations in the concentration of plasma radioiodide resulting from changes in the extrarenal metabolism of iodide (e.g. in inter-/intracellular distribution (11)) but involves a change in functional activity of the kidney, since the clearance of radioiodide was increased. The fact that posterior pituitary hormone also increases the excretion of chloride (12) as well as sodium and potassium (13) suggests that these hormones may influence the urinary excretion of electrolytes in general.

The role of the anterior pituitary on iodide excretion is not clear. While ACTH increased iodide excretion (Table 2) and growth hormone was found by others to have the same effect in hypophysectomized rats (9), it was not demonstrated that the preparations used were pure, and their effect might be due to contamination by posterior pituitary hormones.

Chronic effect of vasopressin

It was possible that, as in the case of mice given sodium chloride in the diet (1, 2), the mice treated for prolonged periods of time with vasopressin might suffer such iodide losses that a relative iodine deficiency would occur which might result in thyroid stimulation. Such a stimulation did not occur. On the contrary, the decrease in the weight and radioiodine uptake of the thyroid gland observed after prolonged treatment with vasopressin indicates that the thyroid activity was depressed. The high mortality (58%) and the loss of weight in the animals administered the hormone indicate that this treatment resulted in general damage or stress, which may account for the thyroid depression. Presumably, this effect prevented the gland from being stimulated by the iodine losses.

In conclusion, the urinary excretion of the iodide ion was significantly increased by the posterior pituitary hormones vasopressin and oxytocin, while it was but little influenced by adrenal hormones.

Acknowledgment

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HEPATIC REGULATION OF ADRENAL CORTICAL FUNCTION[1,2]

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ABSTRACT

Under many different experimental and physiological conditions, a close correlation has been found between the size of the adrenal glands and the capacity of the liver to inactivate adrenal cortical hormones by ring A reduction *in vitro*. We have concluded from this close correlation that there is a similar parallel relationship between the *in vivo* capacity of the liver to inactivate corticosteroids, and the adrenal cortical secretion rate. This conclusion is based on several assumptions which are discussed. The present experiments demonstrate that this relationship denotes secondary responses of the adrenal cortex to primary, independent variations in hepatic inactivation of corticosteroids. Neither ACTH nor adrenal cortical hormones increases the capacity of the liver to inactivate corticosteroids. In contrast, however, a primary diminution of hepatic capacity for adrenal steroid inactivation by surgical removal of 65% of the liver tissue can cause secondary adrenal atrophy. Therefore, the parallel relationship between hepatic capacity for inactivation of adrenal cortical hormones by ring A reduction, and the size of the adrenal glands represents a controlling influence of the liver on adrenal cortical function, rather than the reverse.

This hepatic regulation of adrenal cortical function is apparently mediated through the anterior pituitary (ACTH) negative-feedback control which stabilizes plasma corticosteroid concentration. Our data indicate that the control very effectively matches the mean input of adrenal cortical hormones to any given output over wide ranges of steroid inactivation rates. By means of these data, the automatic control properties of the hepatic-anterior pituitary-adrenal cortical system are analyzed to show that the amount of TPNH-bound Δ^4-steroid hydrogenases in the liver may affect: a) the time constant of the system (the time required to diminish by 63% a deviation from the mean plasma corticosteroid concentration); b) the extent of hunting of plasma corticosteroid concentration about a mean value; c) the half-life of the steroid molecules in the system; d) the velocity of their inactivation at any given plasma corticosteroid concentration; and, e) the mean concentration of adrenal cortical hormones in the blood.

IT HAS been proposed that the concentration of the adrenal cortical hormones in blood is stabilized by a negative-feedback control of adrenal cortical secretion rate, so that changes in the rate of secretion are brought about by opposite changes in the corticosteroid concentration in blood (2,

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[2] A preliminary report has appeared (1).

3). The concentration of these steroids in blood depends upon both the rate of secretion of the hormones into blood, and the rate of removal of them from blood. Since urinary and biliary losses of active corticosteroids are negligible (4, 5), the removal of adrenal steroids is accomplished almost entirely by the enzymatic processes which inactivate the molecules. The main site of corticosteroid inactivation is the liver (6, 7). Therefore, if it is true that the rate of adrenal cortical secretion is regulated by the proposed negative-feedback control, changes in the rate of hepatic metabolism of these hormones should cause parallel changes in the rate of adrenal cortical secretion. The demonstration of this predicted relationship would establish the liver as a principal determinant of adrenocorticotrophic hormone (ACTH) release and adrenal cortical secretion rate in normal animals, and provide new support for the negative-feedback control hypothesis.

Studies of patients with liver or thyroid disease (4, 7, 8) have suggested that the liver exerts some influence on adrenal cortical function. Under a wide variety of physiological and experimental conditions, we have found a remarkably close parallel relationship between the capacity of the liver to inactivate adrenal cortical hormones *in vitro* by ring A reduction, and the size of the adrenal glands (9, 10). Female rats have larger adrenals than males, and their livers have a greater *in vitro* capacity to inactivate corticosteroids (10). In hamsters these hepatic and adrenal sex differences are both reversed: male hamsters have larger adrenals and greater capacity to inactivate corticosteroids than have females (10). Hyperthyroid rats of each sex have both larger adrenals and greater capacity for steroid metabolism by liver than do normal rats of the same sex. In hypothyroid animals both adrenal size and steroid metabolism by liver are diminished. The correlation between adrenal size and the capacity of the liver to inactivate the corticosteroid substrate was extraordinarily close in those experiments ($r = 0.97$) (9).

In addition to the above evidence, we have also found that castration of young rats abolishes both the hepatic and adrenal sex differences. These sex differences can then be re-established together by treating the castrated males with androgens, and the castrated females with estrogens, or be completely reversed together by giving the estrogen to the castrated males, and the androgen to the spayed females (10). Furthermore, in both sexes of caesarian-delivered rats raised on a pasteurized chow diet, the livers comprise a larger fraction of total body weight, and have higher Δ^4-steroid hydrogenase activity than is usual for the strain after normal birth and growth on an ordinary chow diet. The relative adrenal size of such animals is also larger than is usual for the strain (unpublished data). Finally, even the rapid adrenal enlargement of the female rat at estrus is associated with a parallel increase in total hepatic Δ^4-steroid hydrogenase activity, as shown in the experiments to be described.

From this very clear relationship between the *in vitro* capacity of the liver for the reduction of adrenal cortical steroids at ring A, and the size of the adrenal glands, we have concluded that there is a similarly close relationship between the *in vivo* capacity of the liver to inactivate adrenal cortical hormones, and the secretion rate of the adrenal cortex. This conclusion is based on three assumptions, in addition to the evidence presented above. The assumptions are: a) ring A reduction is a major reaction leading to inactivation of corticosteroids in liver; b) changes in total adrenal weight are an indication of parallel changes in adrenal cortical secretion rate in the conditions and species studied; and c) judgments about hepatic metabolism of adrenal steroids *in vivo* can be made from these *in vitro* assays. Insofar as these assumptions are true,[3] a close parallel relationship between hepatic capacity for inactivation of corticosteroids and adrenal cortical secretion rate appears to be established.

Before this close relationship can be taken as evidence for the hepatic regulation of adrenal cortical function, it must be shown that primary changes in hepatic capacity for corticosteroid inactivation can cause parallel changes in adrenal cortical secretion rate, but that primary alterations of plasma corticosteroid concentration do not cause parallel changes in hepatic inactivation of these hormones. The present experiments were undertaken to test these two propositions, and through them, the prediction that the liver regulates adrenal cortical function.

METHODS

Adult male and female Sprague-Dawley rats weighing 200–500 gm. were used in all experiments. The animals were kept in a thermoregulated room (22° C) and were maintained on Purina chow and tap water.

All assays of hepatic capacity for the *in vitro* inactivation of adrenal cortical hormones were performed in duplicate. Corticosterone was the substrate, liver slices were the source of enzymatic activity, and the loss of spectral absorption of the steroid at 240

[3] The products of ring A reduction are inactive at physiological concentrations (11), and the reaction is for practical purposes irreversible (12). Although reduction of the C_{20} carbonyl group may occur without prior reduction of ring A (13, 14) and is sufficient to inactivate corticosteroids (15), ring A reduction appears to be the first alteration of the steroid molecules in the major *in vivo* pathway for metabolism of adrenal steroids (16). Subsequent reductions of the 3 and 20 ketones, and the conjugation with uridine diphosphoglucuronic acid which occurs in most of the species studied, serve to increase the water solubility of the inactivated molecules and facilitate excretion of them from the body.

Under some experimental conditions, changes in adrenal size do not give an accurate indication of changes in adrenal cortical secretion rate (17). However, adrenal cortical secretion is controlled by ACTH, and from the data of Sayers *et al.* (18) it can be shown that adrenal size is linearly related to the log of the dose of ACTH in hypophysectomized rats. If time for the change in gland size is allowed, the assumption that changes in gland size indicate sustained changes in secretion rate, under most physiological conditions, is reasonable.

mμ was the measure of ring A reduction. Animals were killed by a blow on the head and their livers were quickly excised, rinsed in iced saline, and weighed. Slices were cut on a Stadie-Riggs hand microtome, and 200 ± 5 mg. of slices were added to each incubation flask containing 2 ml. of warmed, oxygenated phosphosaline buffer (19). Each 2 ml. of the buffer contained 18.2 μM glucose, 0.368 μM corticosterone, and a trace (0.4%v/v) of ethanol.

The reaction mixtures were incubated for 15 minutes at 37.5° C under 100% oxygen at ambient pressure, with continuous shaking. Similar mixtures were held at 0° C for 15 minutes. The reactions were stopped and the extraction of unmetabolized steroid was begun by the addition of 20 ml. of cold redistilled dichloromethane to each vessel. Thereafter, the procedures were exactly as described previously for experiments with liver homogenates (20). Recovery of known amounts of added steroid from the 0° C mixture was 98% complete. The background readings contributed 13% of the total optical density of the 0° C mixture at 240 mμ.

The reaction velocity in these assays is very dependent upon the initial substrate concentration, and it was necessary to reproduce this concentration very accurately in every experiment. In spite of this difficulty we have found that the slice assays are preferable to homogenate assays, in which zero-order kinetics can be achieved (20), for the purposes of these experiments. The reaction velocity in the slices is affected by changes in the availability of both the enzyme and the coenzyme, TPNH (reduced triphosphopyridine nucleotide) (9), involved in the reaction. The homogenate assay can accurately measure only variations in the amount of the enzyme. We have previously shown that the amounts of enzyme and available coenzyme may be changed independently under various experimental conditions (9). For this reason the slice assay gives a better *in vitro* estimate of the *in vivo* effect of a treatment than does the homogenate assay.

The expression of total hepatic capacity for the *in vitro* reduction of corticosterone at ring A is obtained by multiplying the amount of metabolism in 200 mg. of slices in 15 minutes (the activity concentration) by

$$\frac{(5)(\text{total liver weight in gm.})(100)}{(15)(\text{body weight in gm.})}.$$

The final activity unit is therefore: μM corticosterone reduced at ring A per minute per 100 gm. body weight. This form of expression for the total hepatic capacity for steroid ring A reduction is independent of any quantitative change in liver tissue constituents, such as glycogen, which may have occurred during an experiment. An increase in liver glycogen, for example, would increase liver size, but the accompanying decrease in activity concentration would leave the calculation of total capacity unaffected.

For interpretation of the results it is necessary to distinguish between the hepatic capacity for corticosteroid inactivation, as calculated above, and the instantaneous rate of hepatic inactivation of the hormones. The hepatic capacity is a measure of the hepatic content of active enzyme (enzyme plus necessary cofactors) involved in the metabolism of the steroid molecules. The instantaneous reaction rate *in vivo* depends both upon this capacity, and upon the existing substrate concentration in the blood, since the removal of corticosteroids from blood is a first-order process (21, 22). An increase in blood concentration of adrenal cortical hormones would be followed by an increased rate of removal of them from blood, even at constant hepatic capacity for adrenal steroid inactivation. The hepatic capacity, however, determines the reaction rate for any given substrate concentration, and also determines the half-life of the hormones at all concentrations.

Animals treated with ACTH received 10 U./day of Organon ACTH dissolved in

1 ml. of gelatin diluent (16% gelatin, 30% propylene glycol, 0.5% phenol) subcutaneously. Animals treated with cortisone acetate received either 1.5 or 3.0 mg./day of the Upjohn microcrystalline suspension subcutaneously. Adrenal enucleation and contralateral adrenalectomy were performed at one stage through bilateral dorsal incisions, under ether anesthesia. The blood clot and necrotic center were removed from the regenerating adrenals at the time of weighing, 6 or 12 days after operation. It was often difficult to separate cortical tissue from debris, and the weights recorded are subject to some uncertainty. Partial hepatectomy with removal of approximately 65% of the liver tissue was accomplished by the technique of Higgins and Anderson (23).

Rats receiving the acute, 4-hour intravenous infusions of corticosterone were prepared 18 hours before the start of the experiment by catheterization of the lateral caudal vein with a polyvinyl catheter, under ether anesthesia. The animals were then placed in comfortable restraining cages in which they had previously been trained to remain quiet while without food or water. At the start of the experiment, the catheters were unsealed without disturbing the rats, and connected to a constant infusion pump delivering approximately 2 μg. corticosterone per minute at a flow rate of 0.05 ml./minute of 0.15 *M* saline. This infusion rate was chosen to approximate the maximum rate of secretion of corticosterone by both adrenals in rats of the size employed (24, 25). Control animals received infusions of saline only.

Data presented as means are accompanied by the standard errors of the means.

RESULTS

Control groups: effects of age, sex, and estrus

The liver and adrenals represented larger fractions of body weight in young adult males than in older adult males. The activity concentration was essentially the same in both groups (Table 1), so the total hepatic capacity for steroid metabolism and the relative adrenal weight (total adrenal weight in mg./100 gm. body weight) both appeared to decrease slightly with age. This effect of aging was small and could be ignored as long as all experimental animals weighed between 200 and 500 gm.

Female rats had larger adrenals and greater total hepatic capacity for corticosterone ring A reduction than did males (Figs. 1, 2). During estrus, both the adrenal size and total hepatic activity became still greater. At estrus, the adrenal weight increased by 20%, and the total hepatic capacity for steroid inactivation increased by 23% over the levels at diestrus, through an increase in liver size at constant activity concentration (Table 1; Fig. 2, controls). Since the proportion of females in estrus to those in diestrus is small in a random population of rats, no attempt was made to select animals for the experimental groups according to their stage in the cycle. Results obtained in experimental groups of females are compared to the diestrus control group.

Hyperadrenalcortical groups

Prolonged hyperadrenalcortical states were produced in each sex in two different ways. One group of animals was treated for 12 days with ACTH. These animals showed the expected adrenal hypertrophy, and presumably

TABLE 1. RAW DATA SHOWING EFFECTS OF VARIATIONS IN ADRENAL CORTICAL STATE IN BOTH SEXES

Group		No. of rats	Duration (days)	Body wt. (gm.) Initial	Body wt. (gm.) Final	Liver weight (gm.)	Total adrenal weight (mg.)	Activity concentration (μM corticosterone reduced in 15 min. by 200 mg. of slices)
				Males				
Controls	Young adult	5		204± 5		9.5±0.4	34.0± 2.1	0.12±0.01
	Adult	5		478±24		17.9±1.0	63.3± 4.8	0.14±0.01
	Sham-op.	4	6	357± 7		12.8±0.4	47.4± 6.5	0.17±0.003
Hyper Adrenal Cortical	ACTH 10U./day	5	12	425±8	425±28	16.0±0.2	89.5± 4.6	0.10±0.01
	Cortisone Acetate 1.5 mg./day	5	12	311±2	350±10	14.2±0.5	35.7± 1.7	0.09±0.01
Hypo Adrenal Cortical	With Weight Loss	5	6	412±5	400± 6	14.5±0.5	12.4± 3.2	0.08±0.01
	With Weight Gain	5	6	357±9	367± 7	13.4±0.6	18.2± 3.6	0.16±0.01
				Females				
Controls	Diestrus	6		241± 7		7.5±0.7	60.3± 2.8	0.22±0.01
	Estrus	4		236± 9		8.6±0.3	72.0± 6.3	0.23±0.02
Hyper Adrenal Cortical	ACTH 10U./day	5	12	248±5	253± 8	10.0±0.5	142.4±28.7	0.18±0.01
	Cortisone Acetate, 1.5 mg./day	4	12	268±5	275± 5	9.2±0.3	54.2± 3.6	0.20±0.01
	Cortisone Acetate, 3.0 mg./day	4	12	289±2	270± 6	8.5±0.1	40.8± 3.2	0.19±0.01
Hypo Adrenal Cortical	6 days Post-op.	5	6	242±3	243± 4	8.2±0.3	6.0± 1.3	0.22±0.003
	12 days Post-op.	3	12	252±3	265± 2	9.7±0.1	15.0± 0.9	0.21±0.01

had high blood levels of both ACTH and corticosterone. Another group was treated for the same period with cortisone acetate. These animals showed the expected adrenal atrophy, and presumably had low blood levels of ACTH and high blood levels of cortisone. As shown in Figures 1 and 2, the hyperadrenalcortical state, however produced, did not cause an increase in total hepatic capacity to inactivate corticosteroids in any instance. In fact, a substrate depression of the Δ^4-steroid hydrogenase activity was revealed (Fig. 1, ACTH and cortisone groups; Fig. 2, 10.4 mg./kg./day cortisone group).

In males, the ACTH treatment caused a 30% diminution of total hepatic activity for the reduction of ring A of corticosterone. The treatment with cortisone caused a 26% loss of activity for the metabolism of corticosterone, and a 24% loss for the metabolism of cortisone itself (not shown). In females, neither the ACTH treatment, nor the 1.5 mg./day dose of cortisone (5.6 mg./kg./day) affected the enzymatic activity in the liver for the inactivation of either corticosterone or cortisone. At higher doses (10.4 mg./kg./day) cortisone caused a 14% loss of hepatic activity for the reduction of corticosterone, and a 36% loss for ring A reduction of cortisone (not shown). The dose of cortisone acetate required to reveal the substrate depression of hepatic activity was slightly more than 2 times greater in the

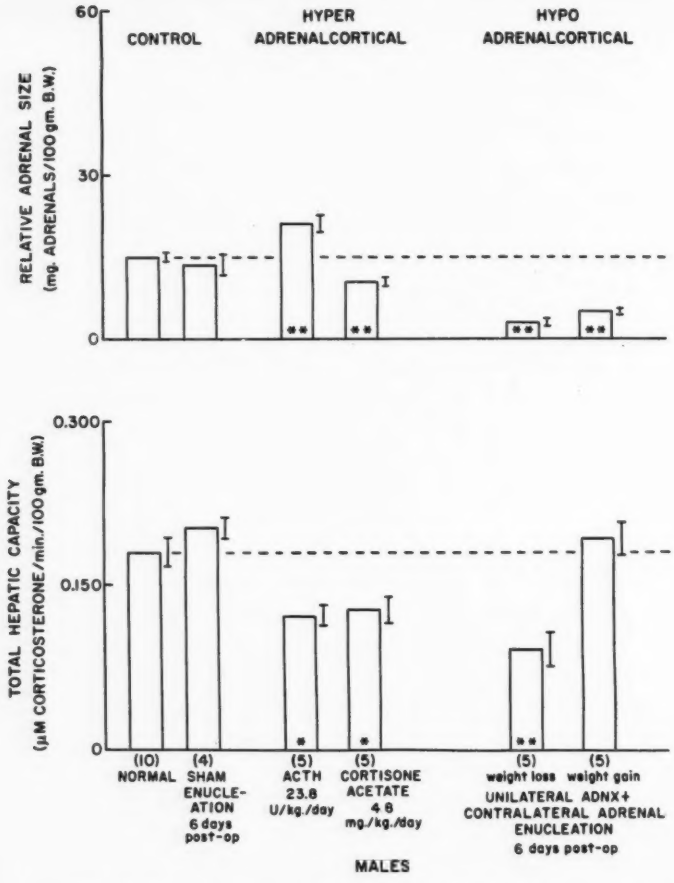

FIG. 1. Derived data showing effects in males of variations in adrenal cortical state on the hepatic capacity for reduction of ring A of corticosterone *in vitro*. Figures in parentheses indicate number of animals studied. Lines beside bars indicate standard errors of the means. The data from young adult and adult control groups were pooled. (*) indicates that the difference between the experimental value and the control value is significant at the 5% level; (**) indicates significance at the 1% level.

female (10.4 mg./kg./day) than in the male (4.8 mg./kg./day), presumably because of the greater capacity of the female to inactivate adrenal cortical hormones (10).

The acute hyperadrenalcortical state produced in four male rats by intravenous infusion of corticosterone at approximately 2 μg./min. for 4 hours also failed to cause any increase in total hepatic capacity for steroid ring A reduction. In fact, a 30% depression of activity by the substrate was ob-

served ($P<0.01$). The control saline infusion of 3 rats had no effect on the Δ^4-steroid hydrogenase activity of the liver.

Hypoadrenalcortical groups

In males and females the hypoadrenalcortical state was achieved by complete removal of one adrenal, and removal of all but a fragment of capsule of the opposite adrenal. The enucleation was done alternately on right

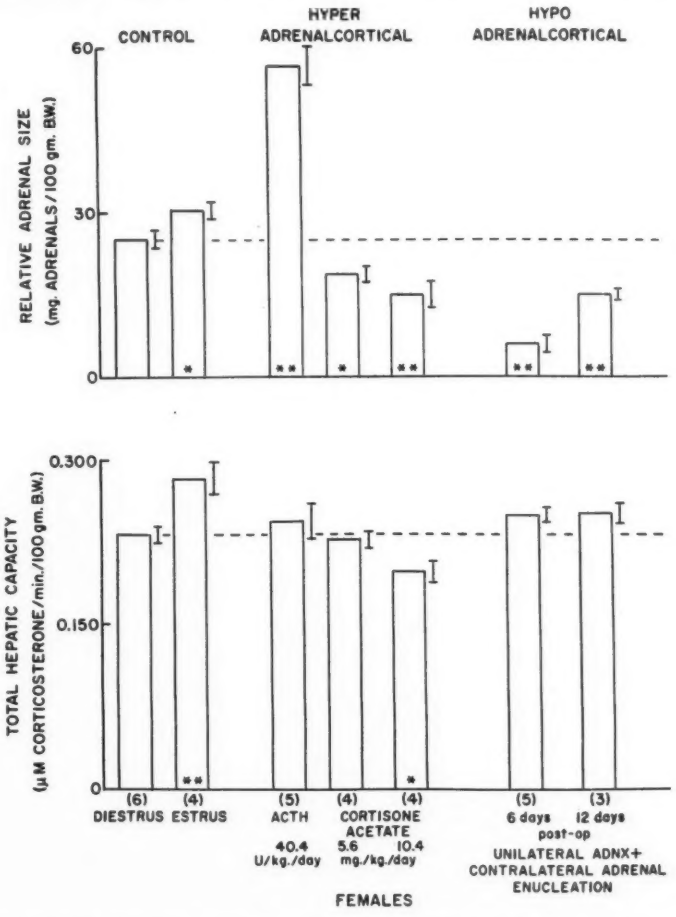

FIG. 2. Derived data showing effects in females of variations in adrenal cortical state on the heptatic capacity for reduction of ring A of corticosterone *in vitro*. Figures in parentheses indicate number of animals studied. Lines beside bars indicate standard errors of the means. (*) indicates that the difference between the experimental value and the diestrus control value is significant at the 5% level; (**) indicates significance at the 1% level.

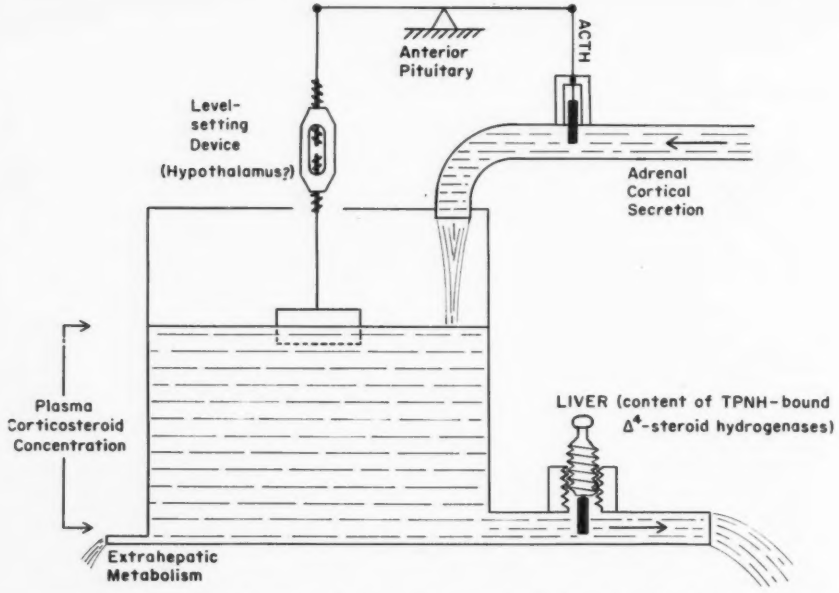

FIG. 3. Hydraulic analogue of the hepatic control of adrenal cortical secretion rate. The hepatic-anterior pituitary-adrenal cortical system can be represented schematically as a single capacity plant with proportional input regulation and proportional output self-regulation (via the first order reaction kinetics *in vivo*) at each setting of the independently variable output regulator. The instantaneous rate of outflow (inactivation), to which the rate of inflow (secretion) will be matched, is determined both by the setting of the output regulator (the total hepatic content of TPNH-bound Δ^4-steroid hydrogenases) and by the fluid level in the system (the concentration of corticosteroids in the blood).

The time constant of such a system is derived from integration of two first order differential equations, one for the inflow relation and one for the outflow relation, and is given by:

$$T = \frac{V}{S + C}$$

where T is the time it takes to diminish by 63% $[1-(1/e)]$ a deviation of plasma corticosteroid concentration from the mean value; V is the volume of distribution of adrenal cortical hormones at plasma concentration; S is the sensitivity of the input regulator; and C is the hepatic content of TPNH-bound Δ^4-steroid hydrogenases.

The liver will therefore have a controlling influence on both the rate of adrenal cortical secretion and the time required to correct an alteration in plasma corticosteroid concentration, regardless of the absolute value of the concentration. In addition, the liver may also influence the mean regulated level of corticosteroids in the blood. In the hydraulic analogue, the mean level (P_m) is given by:

$$P_m = \frac{q_{i\,max}}{S + C}$$

where $q_{i\,max}$ is the rate of inflow which would be observed if the level were zero. Hence,

and left sides. These hypoadrenalcortical animals required no therapy, and the regeneration of the adrenal tissue followed the course described by Ingle (26). Total adrenalectomy was rejected as an experimental test of the effects of decreased adrenal cortical secretion rate on steroid metabolism by liver because prolonged absence of adrenal cortical secretion does not occur physiologically.

The assays were performed 6 days after the surgical removal of most of the adrenal tissue. At that time, only 26% and 42% of the normal amount of cortical tissue was present in two groups of males, and only 26% was present in the females, assuming that the medulla represents 9.2% of the total weight of the normal male adrenal and 6.8% of the total weight of the normal female adrenal (27). The results in the two groups of hypoadrenalcortical males are shown in Figure 1. In the male group which received the more extensive removal of adrenal tissue, all the animals lost weight during the 6-day post-operative period. In these males, which apparently had severe adrenal cortical insufficiency, there was a significant loss of hepatic capacity to inactivate corticosterone by ring A reduction (Fig. 1, hypoadrenalcortical group on left). In the second group, however, with somewhat less extensive loss of adrenal tissue there was no change in hepatic steroid metabolism (Fig. 1, hypoadrenalcortical group on right), and every one of these animals gained weight during the 6-day post-operative period, although at a slower than normal rate. A similarly prepared group was followed for 12 days after operation (not shown). Further adrenal regeneration was found, as described by Ingle (26), suggesting that the concentration of ACTH in blood was still elevated during the 6th to 12th day because of persisting low plasma corticosteroid concentration. The continuing hypertrophy of the adrenals indicates that all the animals assayed at 6 days were really in a hypoadrenalcortical state.

Sham operations consisting of bilateral excision of pieces of perinephric fat were without effect on either adrenal size or hepatic metabolism of corticosterone (Fig. 1, controls).

←

the greater the outflow capacity, the lower the mean regulated level. Changes in the mean regulated level, as a result of changes in outflow capacity, are known as load-errors. In the biological system we have shown that as the hepatic capacity (C) increases, the adrenals hypertrophy, and so compensating changes in $q_{i\,max}$ may minimize the effects of changes in hepatic capacity on the mean plasma concentration of adrenal cortical hormones. Thus, the load-error effect in the biological system may be much less than the hydraulic analogue would indicate.

The high ACTH concentration in the blood of adrenalectomized animals is further increased when the animals are exposed to stressful stimuli (37). Such an effect in animals with zero corticosteroid concentration must have been brought about by some independent variable other than the liver (e.g., hypothalamic excitation). The additional independent variable is represented in the figure by the turnbuckle.

The hydraulic analogue is based upon Smith (29).

Similar results were obtained in the females made hypoadrenalcortical in the same manner, by surgical removal of most of the adrenal tissue, followed by 6 to 12 days of recovery and partial adrenal cortical regeneration. As shown in Figure 2, the hypoadrenalcortical state in females had no effect on the capacity of the liver to inactivate corticosterone by ring A reduction. Considerable adrenal regeneration occurred between the 6th and 12th day, and so it seems likely that the 6-day group was indeed in a hypoadrenalcortical state at the time of assay.

Partial hepatectomy

The results of all these direct attempts to simulate wide variations in adrenal cortical function indicate that changes in the rate of adrenal cortical secretion do not cause parallel changes in the capacity of the liver to inactivate corticosteroids.

TABLE 2. EFFECT OF REMOVAL OF 65% OF THE LIVER ON THE WEIGHT OF THE ADRENAL GLANDS. SPLENECTOMY WAS USED TO CONTROL THE EFFECTS OF ETHER ANESTHESIA AND SURGICAL TRAUMA ON ADRENAL WEIGHT

Group	Body wt. at operation (gm.)	Wt. tissue removed (gm.)	24 hours post-op.		48 hours post-op.		72 hours post-op.		96 hours post-op.	
			Liver wt. (gm.)	Adr. wt. (mg.)	Liver wt. (gm.)	Adr. wt. (mg.)	Liver wt. (gm.)	Adr. wt. (mg.)	Liver wt. (gm.)	Adr. wt. (mg.)
Females										
Splenectomy Controls	240±6.5 (5)	0.8±0.08 (5)	7.4 (1)	66.8 (1)	9.2 (1)	72.4 (1)	9.5 (2)	70.2 (2)	9.7 (1)	77.6 (1)
Hepatectomy	250±2.9 (5)	6.1±0.73 (5)	4.4 (1)	101.8 (1)	7.2 (1)	80.0 (1)	7.8 (2)	83.2 (2)	9.1 (1)	88.1 (1)
Males										
Splenectomy Controls	280±2.6 (5)	2.1±0.19 (5)	11.3 (1)	56.1 (1)	11.7±0.02 (4)	55.6±2.0 (4)				
Hepatectomy	281±7 (5)	6.9±0.32 (5)	3.8 (1)	41.4 (1)	6.3±0.4 (4)	41.3±1.1 (4)				

We then attempted the converse experiment of testing whether direct alterations in the amount of liver tissue would lead to parallel changes in adrenal size. For this purpose male and female rats of the caesarian-delivered Sprague-Dawley strain (Charles River Laboratories) were subjected to surgical removal of approximately 65% of their total liver tissue. They were studied between 24 and 96 hours after operation. The weights of the regenerating liver and of the adrenals were measured and compared to the liver and adrenal weights of animals of the same size which had received total splenectomy as a control operation at the time of the hepatectomy in the others. The results are shown in Table 2.

The adrenals of partially hepatectomized males were atrophic, compared to those of their sham-operated controls ($P < 0.01$) 48 hours after operation, in spite of the fact that more surgical trauma was involved in the hepatectomy than in the splenectomy. Approximately three times more liver tissue than spleen tissue was removed. In the females the effect of partial hepatec-

tomy on adrenal size could not be examined, because the liver regenerated almost completely while the adrenals were still hypertrophied as a result of the stress of the operation. The results in the males, however, show that primary changes in hepatic capacity for inactivation of adrenal cortical hormones can cause parallel changes in adrenal size.

Effects of stress

The demonstration of a very rapid substrate depression of the enzymatic inactivation of corticosteroids by ring A reduction during the four-hour intravenous infusion of corticosterone, suggested to us that this effect of corticosteroids on the liver might reinforce the hyperadrenalcortical state of stress at a time when the plasma concentration of adrenal cortical hormones is high. We studied various types of experimental conditions which are commonly thought to provoke "stress" responses in rats (cold, fasting, trauma, ether anesthesia, exercise) and found that in most instances the ability of the liver to metabolize steroids by ring A reduction was severely diminished. In no case was it increased. These results will be reported in detail elsewhere.

DISCUSSION

The results of the present experiments show that changes in plasma conconcentration of ACTH or adrenal cortical hormones do not cause parallel changes in the hepatic capacity for the inactivation of corticosteroids by ring A reduction. Only in the extreme case of the male group with adrenal cortical insufficiency and weight loss was the change in steroid metabolism in the same direction as the change in adrenal cortical state. Therefore, the close parallel relationship between adrenal size and hepatic metabolism of adrenal steroids previously described does not represent a secondary response of the liver to primary changes in adrenal cortical secretion rate. In contrast, however, independent alterations of hepatic capacity to inactivate adrenal cortical hormones can cause secondary changes in adrenal size. The adrenals of partially hepatectomized male animals were significantly smaller than those of their sham-operated controls (Table 2). From these data we have concluded that the many examples of parallel changes in adrenal size and hepatic capacity to inactivate corticosteroids demonstrate an hepatic control of adrenal cortical secretion rate.

The controlling influence of the liver on adrenal cortical function could be mediated by the mechanism proposed in the negative-feedback hypothesis. According to this hypothesis, the release of ACTH from the anterior pituitary is inhibited by adrenal cortical hormones (2, 3). As a consequence of this inhibition, changes in the plasma concentration of corticosteroids cause opposite changes in adrenal cortical secretion rate, so that the concentration tends to be restored to its initial level. Concentration changes brought about by the liver could signal compensating changes in adrenal cortical secretion rate through such a mechanism, as shown

schematically in Figure 3. After hypophysectomy, the controlling influence of the liver on adrenal cortical function appears to be lost. Hypophysectomy in the rat prevents the adrenal hypertrophy of hyperthyroidism (29, 30), and abolishes the sexual dimorphism of the adrenal glands (31), both of which we have considered to be examples of the controlling influence of the liver on adrenal cortical secretion rate.

The extraordinarily close correlation ($r = 0.97$) between adrenal size and the capacity of the liver to reduce the Δ^4 group of corticosteroids (9) suggests that the negative-feedback control very effectively matches the mean input of adrenal cortical hormones to any given output over a wide physiological range of steroid inactivation rates. Through this feedback mechanism, variations in the hepatic capacity for adrenal cortical hormone inactivation may regulate the rate of ACTH release, as well as the rate of secretion of adrenal cortical hormones. Therefore, in any physiological state in which the liver maintains this controlling influence on adrenal cortical secretion rate, no valid estimate of changes in plasma corticosteroid concentration can be made solely from changes in plasma ACTH concentration, adrenal size, or adrenal cortical secretion rate.

Alterations in the hepatic capacity to inactivate adrenal cortical hormones may affect the amplitude of the hunting of the plasma corticosteroid concentration about a mean value. For example, ACTH release may be influenced by the rate of change of plasma corticosteroid concentration as well as by the absolute magnitude of the change, just as the magnitude of the change in stimulus intensity required to elicit a response from thermal and stretch receptors is inversely related to the rate of the change (32, 33). If the rate of hepatic inactivation of corticosteroids is diminished, a substantial decrement in plasma corticosteroid concentration might occur before the steroid inhibition of ACTH secretion is decreased, and adrenal cortical secretion begins to re-establish the initial concentration. Conversely, if the rate of steroid inactivation is elevated, a very small decrement in concentration might serve to provoke the compensatory response from the anterior pituitary and adrenal cortex. This proposed inverse relationship between hepatic capacity for steroid inactivation and the amplitude of variation of the plasma concentration of corticosteroids about a mean value may explain the difference in variability of plasma corticosterone levels found among groups of male, diestrus-female, and estrus-female rats (34). The coefficients of variation about the means were 34.8%, 14.8%, and 8.6%, respectively.[4] The hepatic capacities for inac-

[4] We derived the coefficients of variation from the data presented as means and standard errors of the means. An "F" test showed that all differences among the standard deviations of the means of the three groups were significant ($P < 0.01$) although the means themselves were almost identical and the groups with the smallest variability contained the fewest animals.

tivation of corticosterone which we have measured in similar groups are 0.17, 0.23, and 0.28 μM/minute/100 gm. body weight, respectively (Figs. 1 & 2).

Although our results indicate that the liver is a principal determinant of ACTH release and adrenal cortical secretion rate in normal animals, other independent determinants of ACTH release may supersede the hepatic regulation of adrenal cortical function under abnormal conditions such as stress. These other influences on ACTH release (e.g. hypothalamic excitation) can cause sustained elevations of adrenal cortical secretion rate which are independent of any changes in hepatic metabolism of the steroid hormones (Table 2, females). The failure of either ACTH or adrenal cortical hormones to increase the capacity of the liver for corticosteroid inactivation indicates that any metabolic effects following such sustained elevations of corticosteroid inflow cannot be abolished by the liver. Increased hepatic capacity for corticosteroid inactivation is therefore not involved in the unexplained escape of animals from the initial sodium and water retention, observed under certain conditions, during treatment with large doses of ACTH or adrenal cortical hormones (35, 36).

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NOTES AND COMMENTS

THE EFFECT OF ACTH ON ADRENAL ASCORBIC ACID IN LAYING HENS

ABSTRACT

In experiments on the effect of ACTH-administration to one-year old laying hens and three-month old pullets, the following results were obtained:

In one-year old laying hens adrenal ascorbic-acid was depleted by 57.0% as compared to their controls. However, no effect of ACTH could be elicited in three-month old pullets.

These results suggest, therefore, that the failure of other workers to obtain a depletion in adrenal ascorbic-acid upon ACTH-treatment may be attributed to the age factor.

Ascorbic acid depletion in the adrenals of animals as a result of corticotropin administration appears to be reasonably well established (1, 2). This fact is explained by the biogenesis of the corticosteroids. Several workers reported that in this respect, the behavior of the avian adrenal gland is quite different from that of other animals, since they failed to observe ascorbic acid depletion in the adrenals of chickens (3, 5, 4), ducks (6), and quails (7) after ACTH administration.

The particular status of birds concerning the elicitation of this reaction cannot be explained on the basis of the slightly different histological appearance of the fowl's gland; the authors, therefore, found it worthwhile to reinvestigate the problem.

MATERIALS AND METHODS

Twelve one-year old White Leghorn laying hens, and twelve three-month old pullets of the same breed were used in these experiments. The birds were divided into 4 equal groups, i.e. six chickens per group. Groups I and II consisted of laying hens, and Groups III and IV of pullets. Each bird in Groups I and III was given a 0.5 ml. intravenous injection of 10 I.U. of ACTH, while the birds in Groups II and IV served as controls, receiving each 0.5 ml. of saline intravenously. The ACTH used was Armour "ACThar" corticotropin, lot number S/43901. All injections were performed exactly one hour before sacrificing the birds for the subsequent removal of their adrenals. The birds were killed by cervical dislocation, and the two adrenals of each bird were weighed together, and their combined ascorbic acid was determined. The method used for ascorbic acid determination was that of Roe and Kuether (7), using Norit as an oxidizing agent. Colorimetric readings were made on a "Hilger" electrophotometer.

RESULTS AND COMMENTS

As may be seen from the table, the average absolute amount of adrenal ascorbic acid in the non-treated laying hens (Group II) was approximately 30% lower than that of the pullets. The ascorbic acid concentration of the pullets' glands was twice that of the hens, due to the smaller weight of the pullets' adrenals. ACTH injections in the laying hens (Group I) caused an average depletion of 57% in their adrenal ascorbic acid content. At the same time, no effect of ACTH treatment on adrenal ascorbic acid depletion could be detected in the 3-month old pullets.

In view of the lack of effect of ACTH injections on the adrenal ascorbic acid of immature chickens, and in view of the positive results obtained with older birds in our experiments, the following conclusions may be drawn:

1. The failure of other workers to observe a depletion of adrenal ascorbic acid in birds following ACTH injections may be attributed to the age factor, as their experiments

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EFFECT OF ACTH ON ADRENAL WEIGHT AND ASCORBIC ACID CONTENT

	Mean adrenal weight (mg.)	Mean ascorbic acid content (mg.%)	Mean ascorbic acid concentration (mg. %)
Group I ACTH-treated Laying Hens	135 ± 11.00	95 ± 7.40	70 ± 5.00
Group II Saline-treated Laying Hens	138 ± 14.00	222 ± 19.00	161 ± 18.20
% Difference between Groups I and II		57%	56%
Group III ACTH-treated Pullets	94 ± 3.00	309 ± 13.20	329 ± 11.60
Group IV Non-treated Pullets	91 ± 2.40	305 ± 2.78	335 ± 7.00
% Difference between Groups III and IV		1.3%	1.9%

were carried out on 2-day old chicks (3), 37-day old chicks (4), 6- to 10-week old cockerels (5), 23-day old ducks (6), and 12-week old quails (7).

2. In older birds, the adrenals respond to ACTH treatment with the same sensitivity as mammalian adrenals, when judged by the depletion of ascorbic acid content. Similarly, age differences have been observed in the chicken in connection with other phenomena linked to adrenal activity. Thus, while a single injection of adrenal cortical extract (ACE) in the adult fowl caused a transient lymphopenia, and leucocytosis (9), ACE in the chick embryo caused no change in lymphocytes, but a rise in polymorphonuclear cells (10). ACTH in the 5-day old chick had no effect on the number of eosinophils (11).

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INFLUENCE OF ANDROGENS ON ENZYMES OF THE KREBS CYCLE[1]

ABSTRACT

The concentration and total fumarase activity of mouse but not rat or guinea pig liver was decreased by castration and maintained or restored to normal by testosterone administration. The activities of succinic, malic and isocitric dehydrogenases of the liver were not altered in any of the species. The concentration of activity of none of the enzymes was altered in the kidney, heart or muscle. Thus, those tissues which changed in weight after castration and androgen administration exhibited a change in total enzyme activity in direct proportion with the change in weight of the tissue.

Androgens are able to stimulate the synthesis of new tissue in many organs of the body (1) and also increase the utilization of body fat (1, 2, 3) without an apparent increase in energy metabolism (1). It seemed important, therefore, to determine whether the activity of any of the enzymes of the energy producing Krebs Cycle were altered during these androgen-induced changes in metabolism.

Animals: The rats (Sprague-Dawley) were maintained in individual metal cages, the mice (National Laboratories, Creve Coeur, Mo., or Stokeley-Peterson Co., Madison, Wis.) were kept in groups of 3 or 4 in glass jars containing wood shavings, and the guinea pigs (Hartley strain, Tumblebrook Farms) were kept in groups of 3 to 4 in metal cages with screen bottoms. The rats and mice were fed ad libitum the Rockland Mouse diet and the guinea pigs received the Purina Rabbit Checkers or Superior Rabbit pellets with supplements of 25 mg. of Vitamin C and 30–40 gm. carrots three times per week. The artificial light was regulated at 12 hours per day. The temperature for the rats and mice was maintained at 25–27° C and that for the guinea pigs at 22–23° C.

Castration and subcutaneous implantation of pellets of androgens[2] were carried out under ether anesthesia.

Enzyme determination: The guinea pigs and rats were stunned by a blow on the head and then bled to death by cutting the blood vessels of the neck. The mice were killed by dislocating the spine at the base of the skull and cutting the blood vessels of the neck. The tissues were immediately removed, weighed and an appropriate aliquot placed in ice-cold redistilled water for homogenization in Potter-Elvejhem type all glass homogenizers.

The methods for the determination of succinic dehydrogenase (4, 5), malic dehydrogenase (5), isocitric dehydrogenase (6) and fumarase (7) were as reported. The succinic dehydrogenase experiments with mice were repeated with the Warburg procedure (5) with essentially the same results.

RESULTS

Organ Weights: The changes in organ weights due to castration and androgen treatment were as expected (1, 8).

Fumarase: Mice. Castration did not significantly alter the concentration of fumarase activity in the heart or the kidney of the mouse (Fig. 1). Thus, the activity of the enzyme changed in direct proportion with the changes in weight of these two organs. The activity of fumarase in the liver (Fig. 1), on the other hand, showed a definite decrease in concentration within 10 days after castration and remained low during the period of castration. Furthermore, castration at an older age, resulted in a prompt decrease in the concentration of activity. The implantation of a pellet of testosterone at the time of castration or 60 days later maintained and restored respectively the activity of the enzyme.

Rats and Guinea Pigs: The concentration of fumarase activity of the liver, kidney and heart of rats and guinea pigs was not changed after castration or various doses and duration of androgens.

Malic, succinic and isocitric dehydrogenases: The activities of these enzymes in the

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[1] This investigation was supported by research grants, C-1954 and CY-3566, from the National Cancer Institute of the Public Health Service.

[2] The androgens were generously provided by Syntex and Ciba Pharmaceutical Products, Inc.

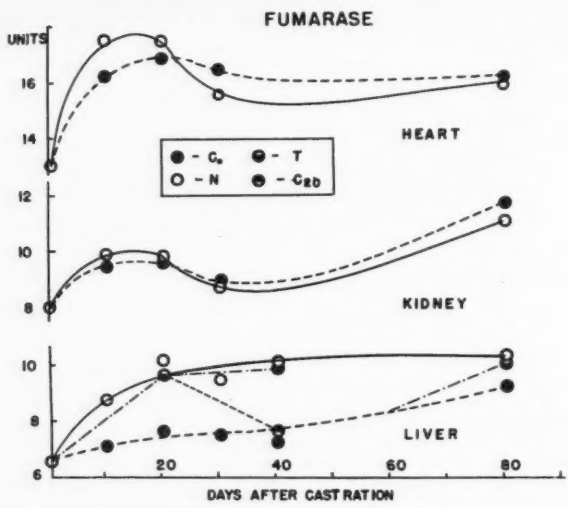

FIG. 1. The effect of castration, testosterone and age on the concentration of fumarase activities in the tissues of the mouse. C_0 = Castration at 35–40 days of age; C_{20} = Castration at 55–60 days of age; T = 15 mg. pellet of testosterone implanted subcutaneously 21 days before autopsy. The units are per mg. wet weight of tissue; 1 unit = a change of 0.01 O.D./min. at 240 mμ. (7).

liver, kidney and heart of the mouse, rat and guinea pig were not affected by castration or androgen administration. Furthermore, the malic and succinic dehydrogenases were not altered in the temporal muscle of the guinea pig or the gastrocnemius muscle of the rat.

Effect of Age: A progressive increase with age was evident in the activity of isocitric dehydrogenase of the mouse tissues especially in the heart and an initial increase in the fumarase activity. The other two enzymes were not studied over a sufficiently long period of time to warrant any definite conclusions.

DISCUSSION

These studies indicate two definite roles of the androgens in regulating the activities of the Krebs Cycle. The first is a general function. These enzymes change in activity in direct proportion with the decrease in protein synthesis after castration and the increase after androgen administration as seen in the kidney and heart of the mouse and rat and the temporal muscle of the guinea pig. Since most of these enzymes are present in the mitochondria, it would seem that the androgens induce synthesis of some elements of the mitochondria at the same time that they are inducing synthesis of other protein constituents of the particular tissue. Several other enzymes are known to change in direct proportion with the changes in weight of these same tissues after castration and androgen administration, e.g., the alanine-glutamic and aspartic-glutamic transaminases of the temporal muscle of the guinea pig (9) and the kidney and heart of the mouse (10), the acid phosphatase of the mouse kidney (11), and adenosine triphosphatase of the kidney and the heart of the rat and the mouse (10). Furthermore, the succinic dehydrogenase (12) and the oxygen consumption (13) of the perineal muscles of the rat also show a directly proportionate change with weight.

It is noteworthy that the decrease in fumarase activity of the liver of the mouse is prevented or restored by testosterone administration. The activity of the enzyme, however, is never increased above normal. Furthermore, the need of androgen to maintain the fumarase at normal activity is peculiar to the mouse liver. Not only the other tissues but also the liver of the rat and guinea pig show no tendency to require androgen for the maintenance of normal fumarase activity.

The reports (14, 15) that castration results in higher succinic dehydrogenase activity in the liver of rats cannot be reconciled with the results of this study.

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THE EFFECT OF EXCESSIVE IODINE ON THE THYROID OF THE RAT

ABSTRACT

The immediate effect of large single doses of iodide on thyroid function in the rat has been studied with the aid of I^{131} and chromatographic analysis. Organic binding of iodide is initially depressed, but the effect wears off after 16 hours presumably as the plasma iodide declines. The ratio of monoiodotyrosine to diiodotyrosine is elevated at the time of minimal binding.

Long term treatment (7 weeks) of rats with iodide results in an initial suppression of I^{131} binding in the thyroid. The effect has disappeared after 3–4 days but iodide binding falls slowly during the ensuing weeks. There is little or no evidence of hypothyroidism in these animals.

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In 1948, Wolff and Chaikoff (1) showed that the organic binding of iodine by the thyroid gland of the rat could be almost completely blocked by the administration of relatively large doses of iodide. This inhibition was related to plasma iodide levels and lasted about 17 hours. It was further shown that the amount of diiodotyrosine (DIT) formed in the gland was smaller, and the conversion of DIT to thyroxine (T_4) was considerably slower when large amounts of iodide were injected (2). However, even if a high plasma level of iodide was maintained, the blocking effect was only temporary, and the formation of significant amounts of organically bound iodide was resumed within 24 hours (3).

The recent findings (4) that the rat thyroid gland could synthesize monoiodotyrosine (MIT) under the influence of propylthiouracil (PTU) and perchlorate, but could not carry thyroid hormone biosynthesis beyond this point prompted us to investigate the nature of the small fraction of organic iodine formed when I^{131} was injected together with a large dose of nonradioactive iodide.

METHODS AND MATERIALS

Adult male hooded rats were fed on the Bruce and Parkes diet 41b (diet 41 (5) omitting cod liver oil and containing a vitamin supplement); the iodine content of the diet is not known. In order to determine the effect of a single dose of iodide each rat was given an intraperitoneal injection of 500 μg. iodide (as KI) together with 100 μc. I^{131}. All experimental groups contained 6 animals. The rats were anesthetized with ether and their thyroids were removed at 1, 2, 4, 8, 16, and 24 hours after the injection. The glands were immediately chilled in a beaker standing in dry ice. In other experiments I^{131} was administered 1 or 2 hours after 500 μg. iodide; the thyroids were removed 4 hours later.

Experiments were also carried out on rats pre-treated with iodide, administered as a 0.01% iodide in the drinking water; in this way each animal received about 1 mg. iodide per day. Thyroid analyses were performed daily for 7 days and at 2, 3, 5 and 7 weeks; each animal was injected with 500 μg. iodide and 100 μc. I^{131}, the thyroids were removed 4 hours later and chilled.

Control rats were injected with 10 μc. I^{131} alone and their thyroids were removed and analyzed at the appropriate time after the injection.

Analysis of thyroid glands

Protein bound thyroidal I^{131} was determined in the manner previously described (4). In some experiments, the radioactivity in the glands was estimated in a well-type scintillation counter before and after dialysis against running tap water. Portions of the glands were hydrolyzed with trypsin in borate buffer at pH 8.6 and the hydrolysates were chromatographed in butanol-dioxan-2N NH_3, 4:1:5 (BDA) and butanol saturated with 2N acetic acid (BAc). The distribution of radioactivity on the chromatograms was measured in an automatic strip counter.

Determination of thyroidal I^{131} uptakes and thyroid: serum iodide (T/S) ratios.

Normal rats and rats pre-treated with PTU (10 mg.), injected intraperitoneally 1 hour before the experiment, were given 0.1 μc. I^{131}; rats injected with 500 μg. iodide received 10 μc. I^{131}. One hour later the thyroids were removed and weighed, and the radioactivity in the glands and sera was measured. The thyroidal PBI^{131} and inorganic I^{131} was measured as described above.

RESULTS

Effect of a single injection of iodide

During the first 2 hours after the injection of 500 μg. iodide and I^{131}, the PBI^{131} was less than 10% of the total thyroidal I^{131}, compared with over 90% in the controls; thereafter it rose steadily and at 16 hours did not differ from that of the controls (Fig. 1).

Chromatographic analysis of thyroid hydrolysates showed that the ratio of I^{131} MIT/DIT in the treated animals was considerably higher than in the controls in the first 8 hours after injection; during this period in the experimental group, only small amounts of labelled DIT could be detected (Table 1). However, after 16 hours the I^{131} MIT/DIT had returned to normal levels. Similar results were obtained when the iodide was administered 1 hour before the I^{131}.

TABLE 1. IMMEDIATE EFFECT OF IODIDE ADMINISTRATION ON THYROIDAL I^{131} MIT/DIT

Time after injection (hours)	I^{131} MIT/DIT	
	Experimentals	Controls
1	4.9	0.86
2	4.2	0.92
4	4.4	0.82
8	3.7	0.53
16	0.66	0.63
24	0.57	0.63

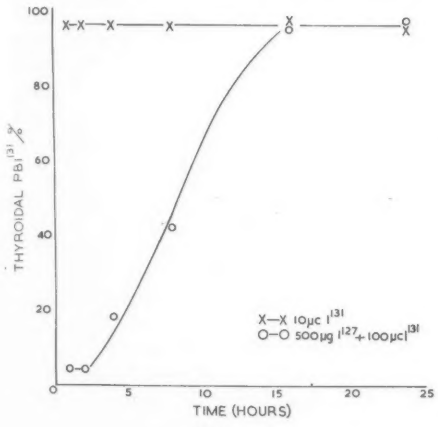

FIG. 1

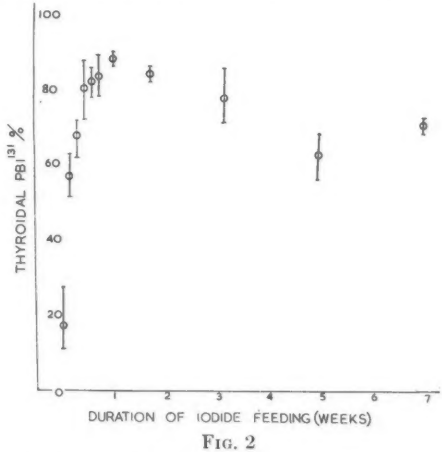

FIG. 2

FIG. 1. Effect of a single large dose of iodide on organic binding of I^{131} in rat thyroid glands.

Open circles represent PBI^{131} as a per cent of the total I^{131} in the glands of rats injected with 500 μg. iodide + I^{131}.

Crosses represent PBI^{131} in glands of control rats given I^{131} alone.

FIG. 2. Effect of chronic iodide feeding (1 mg. per day) on organic binding of I^{131} in rat thyroid glands. Brackets indicate range of value obtained in each group.

Effect of chronic iodide administration

The gradual increase in thyroidal PBI^{131} per cent found in rats fed iodide for increasing lengths of time up to 7 weeks is shown in Fig. 2.

It is evident that the depressed organic binding of iodine in the thyroid which is produced by excessive iodide administration is only temporary, since 85% of the thyroidal I^{131} was protein bound in animals which have been pretreated with iodide for 4 days. Prolonged iodide feeding, however, resulted in a gradual fall in per cent of thyroidal radioiodine present as PBI^{131}. The corresponding I^{131} MIT/DIT ratios are shown in Table 2. As in the short term experiment, this ratio was highest when organic binding of iodine was most depressed; at all times it was somewhat higher than normal.

T/S ratios

In normal rats and in those where organic binding of iodide was blocked with PTU, the T/S ratios varied between 31 to 158. The average hourly thyroidal I^{131} uptake in

TABLE 2. EFFECT OF PROLONGED IODIDE FEEDING ON THYROIDAL I^{131} MIT/DIT

Duration of treatment (days)	I^{131} MIT/DIT
1	1.25
2	1.20
3	0.60
7	0.62
14	0.82
21	0.75
35	0.92

normal animals was 3.3% of the dose, and increased to 10.6% in 24 hours. In the iodide treated rats the T/S ratio varied between 11 and 22; the I^{131} uptake was 0.21% and 0.56% of the dose 1 and 24 hours after the injection.

COMMENT

These findings are in agreement with those of Wolff and Chaikoff (1) who reported that a single excessive dose of iodide exerted an immediate depressing effect on the organic binding of iodine by the rat thyroid. This effect lasted about 16 hours, by which time the concentration of iodide in the plasma had markedly decreased. The present results have also shown that when organic binding of iodide is most depressed, the MIT/DIT ratio is very high. An elevated MIT/DIT ratio has been observed in several conditions where the thyroid might be expected to be iodine deficient (4, 6, 7) or where thyroid function was undeveloped (8), or abnormal (9); it appears surprising that this ratio should be elevated when the gland must be saturated with iodide.

In the rats pretreated with iodide the marked depression of organic binding was only temporary and the gland escaped from this effect after 3–4 days. However, the organic binding appeared to decrease slightly with continued treatment (Fig. 2) although the MIT/DIT ratios were not greatly elevated. The thyroid glands of rats that had been fed iodide water for 7–8 weeks were normal in appearance and weight. Histological examination of these glands showed only a slight increase in follicle size with minimal changes in cell height. Neither did these animals show the usual signs of hypothyroidism such as coarsening and loss of hair and the increased weight found in thyroidectomized adult rats. It appears that the rat thyroid does not respond in the same dramatic way to iodide as does the chick thyroid (10). Whether even more prolonged administration of iodide will eventually induce goiter in rats as it can occasionally do in man (11–19) is at present being investigated.

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A TEST FOR PROLACTIN USING THE HYPOPHYSECTOMIZED EFT PHASE OF *DIEMICTYLUS VIRIDESCENS*

ABSTRACT

Hypophysectomized, terrestrial efts of *Diemictylus viridescens* respond to treatment with prolactin above an estimated threshold of 0.04 I.U. by migrating to water. The protocol for conducting a prolactin assay using the eft is described and the results of an assay of highly purified prolactin are given.

In recent years a minimum dose assay method for the estimation of prolactin has been developed using the terrestrial (red eft) stage of the eastern spotted newt, *Diemictylus viridescens*, as the test organism. A description of the technique is presented here in order that its protocol be available to those interested in the problem.

Following metamorphosis, individuals of *Diemictylus* enter into the terrestrial eft phase which usually lasts for about three years. The animals then return to water and develop the laterally compressed (keeled) tail, lateral line system and olive pigmentation of the adult. Reinke and Chadwick (1) demonstrated that efts receiving implantations of whole adult pituitaries voluntarily entered water within a few days following treatment and assumed adult morphological characteristics. Following injections of 14 to 20 mg. of prolactin (Eli Lilly unidentified lot) Chadwick (2) was able to induce such a "water drive syndrome" in thyroidectomized and gonadectomized efts. However, conflicting evidence was presented by Dr. R. W. Payne (personal communication) who elicited the same reaction with various other pituitary preparations. These results may be attributed to endogenous release of pituitary hormones. In investigations utilizing hypophysectomized efts Grant and Grant (3, 4) have verified prolactin in the role of the active water drive principle, but have shown that it is not directly involved with the production of the range of morphological changes associated with the complete water drive syndrome. There is no difference in the response of efts to prolactin above the 3 μg. level (0.10 I.U.) at which all animals respond according to Grant (5), and unpublished data indicates a threshold at about 1.4 μg. (0.049 I.U.) for highly purified prolactin (C. H. Li).

METHODS

Care of test animals: Efts can be kept for several months in good condition without feeding in plastic containers or aquaria provided with earth and a few dried leaves. Their environment should be kept slightly moist but not damp. The white worm *Enchytraeus* or mutant stocks (vestigial or miniature wing) of *Drosophila* provide adequate food when necessary. For ease of handling and assurance that the newts are well removed from the water drive phase of their natural cycle, test animals should be between 40 to 80 mm. in total length and should weigh in a range from 0.5 to 1.5 gm.

Operation protocol: Following anaesthetization in 0.5 per cent chloretone animals are weighed and measured. Recording of the dorsal spot pattern is convenient for the identification

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TABLE 1. THE WATER DRIVE RESPONSE OF HYPOPHYSECTOMIZED EFTS OF *Diemictylus viridescens* TO VARIOUS DOSE LEVELS OF A HIGHLY PURIFIED PROLACTIN (C. H. LI)†

Number of animals	Average weight in gm.	Average length (mm.)	Total dose		% response	Time to water in hours
			Mg.	I.U.		
6	0.823	32.8	0.4	14	100	139 ± 32.03*
6	0.795	35.7	0.04	1.4	100	123 ± 27.21
6	0.991	38.9	0.004	0.14	100	164 ± 38.62
9	0.712	31.2	0.001	0.035	22.2	121 ± 7.80
6	0.874	35.1	0.0005	0.017	0	

* Standard deviation.

† The author is indebted to Dr. C. H. Li of the Hormone Research Laboratory, University of California, who supplied the prolactin used in these tests.

of individuals. Efts are placed on a board in a slight depression padded with cotton soaked in Ringers solution and are held in place with rubber bands. Working under a dissecting microscope, the lower jaw is depressed and the pituitary area identified through the epithelium of the roof of the mouth. It appears as a small, circular area lying at the base of the triangle formed by the diverging row of vomero-palatine teeth. Using a fine blade, microdissection knife the mucosa of the region is cut and a square is carefully chipped away in the underlying bone revealing the pituitary. The pipette used for the removal of the gland is connected to an aspirator and is provided with a small, lateral hole for the regulation of pressure. When the gland has been removed, the chip of bone is replaced. The wound heals rapidly within a period of several days. Though it is advisable to check the operation by sectioning at the termination of the experiment, complete hypophysectomy is usually indicated when the skin of the animals becomes rough and dark because of their failure to molt in the absence of TSH. Following operations the efts are subject to infections but reasonably good prophylaxis can be obtained by placing the animals in a saline solution of malachite green (1:15,000) and potassium dichromate (1:200) for several minutes. An occasional bath in Achromycin (1:100) is necessitated in some cases. In no case should listless or diseased animals be used in tests and animals should be allowed at least two weeks to come to equilibrium following hypophysectomy.

Injection protocol: Test materials (homogenates, dried preparations, etc.) are prepared in a standard amphibian Ringers and delivered from a 0.25 ml.-capacity tuberculin syringe with a 27 gauge Huber point needle. The eft is held belly up with the hind leg of one side held back against the base of the tail. The needle is introduced into the ventral surface of this leg and pushed forward beneath the skin until it passes into the peritoneal cavity just lateral and anterior to the vent. By using the leg to guide and stabilize the needle, internal injury is kept to a minimum. Although the dose is varied to suit the convenience of the experiment, the volume delivered at each injection should be held constant. Experience indicates that a 0.1 ml. injection of a solution delivered every other day is most practical, although if a single injection is contemplated, 0.2 ml. can be injected into larger newts.

Following injections animals are placed in a container (11×8×4 inches for 8 animals) provided with an inch high cross partition of lucite which serves to divide the chamber into an aquatic and terrestrial area. The latter is provided with clean, aquarium pebbles and several pieces of moistened filter paper to provide cover for the animals. A positive test for prolactin is indicated when the efts voluntarily enter water and remain submerged for at least six hours at a time. Water drive usually occurs from five to twelve days following the initial injection. When whole pituitary preparations are administered, the animals molt frequently and upon entering water progress through the water drive syndrome. Olive pigmentation appears in several weeks, while the keeling of the tail is apparent somewhat later. When pure prolactin or preparations with a very high prolactin content are used, water drive alone is elicited. In this case animals fail to molt properly, although patches of epithelium slough off after the animals have been in water for several days.

In order to obtain quantitative estimates of prolactin content, it is at present necessary to use a minimum-dose assay technique based on a threshold of 1.4 μg. (0.049 I.U.), estimated at causing water drive in 50 per cent of test animals. The red eft test has recently been applied with success on elasmobranch, teleost and primate material and should prove of value in situations where the standard pigeon crop method proves impractical. An example of a typical assay series is given in Table 1.[1]

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[1] These results were obtained in an investigation supported by a research grant, A 1786 (C 1), from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

CHROMATOGRAPHIC ARTIFACTS OBTAINED WITH SODIUM IODIDE-I^{131}

Doctor and Trunnell (1) have recently reported experiments on the effect of storage of sodium iodide-I^{131} and the action of various reducing agents on the products obtained from it during storage. From their results, doubt might be cast on the validity of quantitative aspects of I^{131} metabolism determined in humans or experimental animals since only a proportion of the I^{131} appears to be present as iodide.

It has been observed by many workers that if carrier-free sodium iodide-I^{131} is chromatographed in various solvent systems, a number of radioactive bands appear on the chromatograms. If the same sample of radioactive iodide is chromatographed with a small amount of sodium iodide I^{127}, all the bands except the iodide band disappear. This is found whether the radioactive iodide is fresh or stored. It seems therefore that the apparent breakdown of sodium iodide-I^{131} is artifactual and probably results from the absence of a sufficient number of stable iodide ions to allow it to manifest its usual chromatographic mobilities.

In biological experiments, injection of carrier-free I^{131} into the blood stream or tissues will be followed by its immediate dilution by the iodide present in the body. Further, it will quickly come into contact with tissue reducing agents. Lastly it has been shown by Leblond and Süe (2) and Murray (3) that oxidized iodine in the form of iodate is rapidly reduced to iodide in the body and taken up as such by the thyroid gland. For these reasons, it is to be expected that carrier-free I^{131} will behave as iodide in the body.

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2. LEBLOND, C. P. AND P. SÜE: *Am. J. Physiol.* **134:** 549. 1941.
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[1] Senior Postdoctoral Fellow of the National Science Foundation.

The Endocrine Society

1959 PROGRAM OF THE MEETING OF THE ENDOCRINE SOCIETY IN ATLANTIC CITY

THURSDAY, JUNE 4, 1959

8:00 A.M.—Registration—

I. 9:00 A.M.—THYROID

Carolina Room—Chalfonte

Chairmen: *J. E. Rall and W. Parson*

1. The Effect of Trypan Blue on Thyroid Function in the Rat.
   Takashi Yamada (Introduced by Monte A. Greer).
2. The Effect of Excessive Iodide on the Thyroid of the Rat.
   Valerie A. Galton and *Rosalind Pitt-Rivers.* (Introduced by Sidney Ingbar).
3. Comparison of in Vivo and in Vitro Tissue Effects of Triiodothyronine.
   Roger L. Greif.
4. The Effect of Triiodothyropropionic Acid on Serum Cholesterol and Oxygen Consumption in Myxedema and Euthyroidism.
   Robert D. Leeper (by invitation), *Richard S. Benua, Soichi Kumacka* (by invitation), *Jacob L. Brener* (by invitation) and *Rulon W. Rawson.*
5. Respiratory Stimulation in Young Dogfish by Thyroid Hormone.
   Aubrey Gorbman and *A. W. Pritchard* (by invitation). (Invited paper—20 minutes.)

INTERMISSION

6. Idiopathic Familial Elevation of Thyroxine Binding Protein.
   William H. Beierwaltes.
7. Metabolism of Thyroxine by Human Tissue Slices.
   Edwin C. Albright and *Frank C. Larson* (by invitation).
8. Metabolic Regulation of Organic Iodinations in Thyroid Homogenates.
   George C. Schussler (by invitation) and *Sidney H. Ingbar.*
9. Protein Iodination in Sheep Thyroid Mitochondrial-Microsomal Preparations.
   Leslie J. DeGroot and *Evelyn Carvalho* (introduced by John B. Stanbury).
10. Persistent Thyroid Function After Hypophysectomy.
    O. H. Pearson, A. G. Pazianos (by invitation), *E. Greenberg* (by invitation), and *R. S. Benua.*
11. Effect of Stimulation of the Cervical Sympathetic Nerve on the Thyroidal Release of I^{131}-Labeled Hormones.
    Shiro Iino, Kazuo Shizume and *Shigeo Okinaka* (introduced by Monte A. Greer).

II. 2:00 P.M.—ADRENAL CORTICAL SYNDROMES—STEROID METABOLISM

Carolina Room—Chalfonte

Chairmen: *J. McArthur and A. Bongiovanni*

12. 6 Beta Hydroxylation of Cortisol, a Quantitatively Important Pathway of Metabolism in the Human Newborn.
    E. Colle (by invitation), *R. A. Ulstrom, J. Burley* (by invitation) and *R. Gunville* (by invitation).

13. The Urinary Excretion of Three C-21 Steroids in the Adrenogenital Syndrome.
Alfred M. Bongiovanni, Walter R. Eberlein, and *J. Darrel Smith* (by invitation).
14. The Renal Handling of 17-Hydroxycorticosteroids.
R. O. Morgan (by invitation), *A. J. Blair* (by invitation) and *J. C. Beck.*
15. The Influence of ACTH and Hypophysectomy on the Steroid Conjugating Capacity of the Liver.
David L. Berliner (by invitation), *Charles J. Nabors, Jr.* and *Thomas F. Dougherty.*
16. Direct and Indirect Estimation of Pituitary ACTH Reserves in Man Following Steroid Therapy
Donald A. Holub (by invitation), *Julian I. Kitay, Andrew G. Frantz* (by invitation) and *Joseph W. Jailer.*
17. ACTH Suppression Tests in Patients with Cushing's Syndrome.
Grant W. Liddle.

INTERMISSION

18. Urinary Steroid Metabolites in Hypophysectomized Women During Adrenal Insufficiency and Initial Phases of ACTH Stimulation.
Hildegard Wilson, Mortimer B. Lipsett, and *Lillian C. Butler* (by invitation).
19. Suppression of Urinary 17-Ketosteroids in Virilized Women by Estrogen Therapy.
Raymond C. Mellinger and *Richmond W. Smith, Jr.*
20. Transcortin: Characteristics and Levels in Various Conditions.
Avery A. Sandberg and *W. Roy Slaunwhite, Jr.*
21. Studies on the Mechanism of Elevation of Plasma 17-Hydroxycorticosteroids Induced by Estrogen Administration in Man
Eleanor Z. Wallace and *Anne C. Carter.*
22. Endocrine Effects of Methylenedianiline.
William W. Tullner.
23. Salt Losing Hormone in Hyperkalemic Familial Periodic Paralysis.
R. Klein.

III. 2:00 P.M.—PARATHYROIDS: PITUITARY ADRENAL SYSTEM

Viking Room—Haddon Hall

Chairmen: *G. D. Whedon and D. Nelson*

24. Isolation of Parathyroid Hormone Following Extraction with Phenol.
G. D. Aurbach.
25. Purification of Parathormone B by Countercurrent Distribution.
Howard Rasmussen.
26. Activation of Parathyroid Hormone by Cysteine.
Alexander D. Kenny.
27. Effect of Parathyroid Extract on Bone Matrix and Seromucoid.
William P. Deiss and *Leila B. Holmes* (by invitation).
28. Further Evidence for a Dual Effect of Parathyroid Hormone.
John J. Canary (by invitation), *H. Allan Bloomer* (by invitation), *Gabriel G. Carreon* (by invitation) and *Laurence H. Kyle.*
29. ACTH Secretion in Rats with Hypothalamic Lesions.
A. Brodish.

INTERMISSION

30. Limbic System Influences on the Pituitary-Adrenal Cortical System.
John W. Mason, Walle J. H. Nauta (by invitation), *Joseph V. Brady* (by invitation), and *James A. Robinson* (by invitation).

31. Effect of Diencephalic Stimulation on Adrenocortical and Adrenal Medullary Secretion in the Dog.
William H. Ganong and *Alan Goldfien*.
32. Adrenal Cortical and Medullary Responses to Trauma in Dogs with Isolated Pituitaries.
Richard H. Egdahl (introduced by D. M. Hume).
33. The Early and Late Effects of Hypothalamic Destruction or Removal on Pituitary-Adrenal Activity.
David M. Hume and *Benjamin F. Jackson* (by invitation).
34. Ion-Exchange Chromatography of Pituitary Preparations.
Andrew V. Schally (by invitation) and *Roger Guillemin*.
35. ACTH Releasing Factor Active in the Guinea Pig Test.
M. Sideman (by invitation) and H. Sobel.

FRIDAY, JUNE 5, 1959

8:30 A.M.—Registration—

IV. 9:00 A.M.—ANTERIOR PITUITARY

Carolina Room—Chalfonte

Chairmen: *E. Knobil and F. L. Engel*

36. The Cells of the Human Adenohypophysis in Adrenal Cortical and Thyroid Disorders.
Calvin Ezrin and *Heidi Swanson* (introduced by R. F. Farquharson).
37. A Method for the Quantitative Biological Assay of ACTH in Blood.
Paul L. Munson, Wolfgang Toepel (by invitation) and *Mindel C. Sheps*.
38. Increase in Sugar Permeability in the Rat Adrenal Produced by ACTH.
J. Eichhorn, I. D. K. Halkerston, M. Feinstein, amd *O. Hechter* (by invitation).
39. Contrasting Requirements for the Lipolytic Action of Corticotropin and Epinephrine on Adipose Tissue in Vitro.
J. Earle White (by invitation), *Enrique Lopez* (by invitation) and *Frank L. Engel*.
40. Evidence for Species Specificity of Human, Beef and Rat ACTH Demonstrated by Assay in Vitro.
Julian I. Kitay, Donald A. Holub (by invitation) and *Joseph W. Jailer*.
41. A Comparative Immunological Investigation of Pituitary Growth Hormone from Various Species.
T. Hayashida (by invitation) and *C. H. Li*.

INTERMISSION

42. Growth-Stimulating and Other Effects of Human Growth Hormone (Li) in a Pituitary Dwarf.
Roberto F. Escamilla, John J. Hutchings (by invitation), *William C. Deamer* (by invitation) and *Choh Hao Li*.
43. Endocrine Function in Acromegaly.
George J. Hamwi and *Thomas G. Skillman* (by invitation).
44. The Influence of Hypophysectomy and Growth Hormone on Oxidation of Palmitate-1-C^{14} by the Rat.
M. J. Franklin (by invitation) and *E. Knobil*.
45. Unesterified Fatty Acid Oxidation Rates in Acromegaly, Myxedema, Hyperthyroidism and Diabetes.
Josiah Brown and *Leslie R. Bennett* (by invitation).
46. Evidence for Direct Action of Growth Hormone (GH) on Red Cell Formation.
Albert S. Gordon, Burton S. Dornfest (by invitation) and *Anthony Crusco*.

47. Factors Involved in Production of Suspensions of Free Cells Derived from Hypophyses of Rats.
Griff T. Ross (by invitation) and *Robert C. Bahn.*

1:30 P.M.—ANNUAL BUSINESS MEETING, Carolina Room, Chalfonte

V. 2:30 P.M.—ESTROGENS AND ANDROGENS

Carolina Room, Chalfonte

Chairmen: *R. Huseby and L. L. Engel*

48. The Isolation and Identification of Estrogens from Peripheral Blood with a Practical Method of Analysis.
Georg W. Oertel (by invitation), *Charles D. West,* and *Kristen B. Eik-Nes.*
49. "Free Estriol" in Blood Plasma.
Joseph C. Touchstone and *John W. Greene, Jr.* (by invitation).
50. Effect of Chorionic Gonadotropin (HCG) on Conversion of Estradiol to Estriol by Perfused Human Placenta.
Philip Troen.
51. Physiologic and Biochemical Differences between the Estrogen-Sensitive Transhydrogenase and Estradiol Dehydrogenase.
C. A. Villee, D. D. Hagerman (by invitation) and *J. M. Spencer* (by invitation).
52. The Inhibition of Placental Steroid Mediated Transhydrogenase by 2′ Adenylic Acid.
Nina Hollander (by invitation), *Joseph D. Brown* (by invitation) and *Vincent P. Hollander.*
53. Adrenal Estrogens: In Vivo Conversion of Cortisone to Phenolic Estrogens.
Edward Chang (by invitation) and *Thomas L. Dao.*

INTERMISSION

54. Estrogen Synthesis in a Male Patient with a Feminizing Adrenocortical Carcinoma and Hypertension.
C. D. West, L. F. Kumagai (by invitation), *E. L. Simons* (by invitation) and *P. E. Wall* (by invitation).
55. The Effect of Estrogen on Steroid Levels and Urine.
W. L. Harrmann, I. Schindl (by invitation), and *P. K. Bondy.*
56. The Conversion of Progesterone-4-C^{14} to 21-Hydroxylated Steroids by Normal Rat Testis Stimulated with Human Chorionic Gonadotropin.
Hernan F. Acevedo (by invitation), *Oscar V. Dominguez* and *Leo T. Samuels.*
57. Steroid 21-Hydroxylase in Mouse Testicular Interstitial Cell Tumors.
Oscar V. Dominguez, Robert A. Husby and *Leo T. Samuels.*
58. Studies of Structure-Function Relationships of Steroids: Anabolic Potency of Some Testosterone Analogs.
Herbert A. Burke, Jr. (by invitation) and *Grant W. Liddle.*
59. Influence of Androgen on Prostatic Protein Synthesis.
Wells E. Farnsworth (introduced by Grosvenor W. Bissell).

VI. 2:30 P.M.—PITUITARY HORMONES—NEUROENDOCRINE MECHANISM

Viking Room, Haddon Hall

Chairmen: *J. Everett and A. Gorbman*

THOSE REGISTERED FOR THE MEETING OF THE AMERICAN DIABETES ASSOCIATION WILL BE ADMITTED TO THIS MEETING

60. Lithosperm Inhibition of Blood Pressure Depressor Effect of Oxytocin in the Fowl.

W. R. Breneman, Marvin Carmack, Daniel Overack and *Richard Shaw* (introduced by Aubrey Gorbman).

61. Plasma and Pituitary LH: Detection and Quantitative Bioassay in the Rat Following Gonadectomy.
Albert Parlow (introduced by R. O. Greep).
62. Use of the Red Eft Diamictylus Viridescens in the Assay of Prolactin.
William C. Grant, Jr. (introduced by Aubrey Gorbman).
63. Effects of Captivity on Production of Gonadotrophins in Pintail and Black Ducks and Response to Exogenous Hormones.
R. E. Phillips and *A. van Tienhoven* (introduced by S. L. Leonard).
64. Ovulation in the Domestic Fowl.
R. D. Fraps. (Invited paper, 20 minutes).

INTERMISSION

65. Ovulation After Stimulating the Rat Hypothalamus Late in Diestrus.
J. W. Everett, R. L. Riley (by invitation) and *C. D. Christian* (by invitation).
66. Development of Permanent Abnormal Cycles of Behavior in Rats After Experimental Pituitary Damage.
Curt P. Richter.
67. Hypothalamus and Growth.
S. Reichlin.
68. TSH "Rebound Phenomenon" in Rats After Goitrogen Withdrawal.
S. A. D'Angelo.
69. Pituitary Hormone Production and Release in the Thyroidectomized Rat After Thyroxin Administration.
A. N. Contopoulos, M. E. Simpson (by invitation) and *A. A. Koneff* (by invitation).
70. Subcortical Regions of the Primate Brain Regulating Release of Vasopressin from the Neurohypophysis.
Joseph F. Dingman, Eduardo Gaitan (by invitation), *Akira Arimura* (by invitation) and *Robert G. Heath* (by invitation).

VII. ANNUAL DINNER, Haddon Hall Hotel

E. Perry McCullagh, President, presiding

6:30 P.M.—Cocktails, The English Lounge
Sponsored by the donors of the Awards of the Society:
Ciba, Schering, Ayerst, Upjohn and Squibb. Attendance limited to holders of dinner tickets.

7:30 P.M.—Dinner—The Vernon Room
Presentation of the 1959 Awards of the Society by Charles Kochakian:
The Fred Conrad Koch Award
The Ciba Award
Ayerst Fellowship
Schering Scholar
Upjohn Scholar
President's Address: Dr. McCullagh

SATURDAY, JUNE 6, 1959

8:30 A.M.—Registration—Stair Hall, Haddon Hall Hotel

9:00 A.M.—OPENING SCIENTIFIC SESSION OF THE AMERICAN DIABETES ASSOCIATION WILL BE HELD AT THE HADDON HALL HOTEL IN THE VERNON ROOM. ENDOCRINE SOCIETY BADGE WILL ADMIT YOU TO THIS MEETING.

VIII. 9:00 A.M.—ADRENAL GLAND

Carolina Room, Chalfonte

Chairmen: *M. Grumbach and F. C. Bartter*

71. Stimulatory Effect of Aldosterone and Cortisol on the Work of the Isolated Rat Heart-Lung.
Kathyrn W. Ballard (by invitation) and *George Sayers.*
72. The Hemodynamic Parameter Mediating Increase in Aldosterone Secretion.
Donald S. Gann (by invitation), *Ivor H. Mills* (by invitation) and *Frederic C. Bartter.*
73. Preliminary Characterization of Glomerulotropin.
Gordon Farrell, Avery D. Pratt, Jr. (by invitation), *Laurence S. Harris* (by invitation) and *Robert C. Banks* (by invitation).
74. Changes in Metabolic Activities of Steroid Hormones Associated with Methylation and Hydroxylation in Ring D.
Maurice M. Pechet, Evelyn L. Carroll (by invitation) and *Anne P. Forbes.*
75. Effect of Walker Carcinoma on Steroid Diabetes in Rat.
David J. Ingle (introduced by Dwight J. Ingle).
76. Acute Effects of Adrenalectomy and Hydrocortisone on Glucose Tolerance of Diabetic and Eviscerate Rats.
O. K. Smith and *C. N. H. Long.*

INTERMISSION

77. Influence of Age, Adrenalectomy and Cortisol Administration on the Activity of Hepatic Glutamic-Pyruvic Transaminase.
Homer Harding, Fred Rosen and *Charles Nichol* (introduced by Avery A. Sandberg).
78. The Metabolism of 2 C^{14} Pyruvate in Normal and Adrenalectomized Rats.
William W. Winternitz.
79. Observations on the Role of the Adrenal Gland in the Metabolic Response to Surgery.
Maureen Smith, H. A. F. Dudley, J. S. Robson and *C. P. Stewart* (introduced by Grant W. Liddle).
80. Renal-Cardiovascular Pathology in Aging Rats With and Without Exposure to ACTH and Stressors.
George F. Wilgram (by invitation) and *Dwight J. Ingle.*
81. The Effects of Anoxis on Adrenal Cortical and Medullary Secretion in the Dog.
Alan Goldfien and *William F. Ganong.*
82. Effect of Adrenalectomy and Cortisone on Rate of Eruption of Maxillary Incisors in Female Rats.
L. V. Domm, W. A. Wellband (by invitation) and *J. P. Howard* (by invitation).

IX. 2:00 P.M.—INSULIN AND DIABETES

Carolina Room, Chalfonte

Chairmen: *S. Fajans and A. Renold*

83. Differential Resin Binding of Insulin in Sera from Insulin Responsive and Resistant Diabetic Subjects.
Marvin L. Mitchell and *Mary E. O'Rourke* (by invitation).
84. Serum Insulin-Like Activity in Patients with Pancreatic Islet-Cell Tumors
Yves M. Dagenais (by invitation), *Albert E. Renold, Donald B. Martin* (by invitation) and *Vilma Lauris* (by invitation).

85. Role of Individual Organs in Carbohydrate Metabolism in Intact Normal Dogs.
Dorothy H. Henneman, William Shoemaker (by invitation) and *William Walker* (by invitation).

86. Hepatic Glucose Metabolism in Two Forms of Spontaneous Hyperglycemia.
Gaston R. Zahnd, Jean Christophe and *George Yerganian* (introduced by Albert E. Renold).

87. Carbohydrate Metabolism in Amphibians and Reptiles.
Malcolm R. Miller (Introduced by Frank L. Engel). (Invited paper, 20 minutes.)

INTERMISSION

88. On the Mechanism of the Blood Glucose Lowering Action of Insulin.
N. Altszular, A. Dunn (by invitation), *R. Steele, D. T. Armstrong* (by invitation), *J. S. Bishop* (by invitation) and *R. C. de Bodo.*

89. An Insulin Effect on Peptide Synthesis Independent of Glucose or Amino Acid Transport.
Ira G. Wool and *M. E. Krahl* (by invitation).

90. Comparison of Epinephrine and Insulin on Adipose Tissue Metabolism.
George F. Cahill, Jr., Bernard Leboeuf (by invitation), and *Robert Flinn* (by invitation).

91. The Action of Insulin, Tolbutamide and Hypoglycin A on the Adipose Tissue of Rats.
Thomas C. Smith, Lena Will (by invitation), and *Ira Ringler.*

92. Relation of Insulinogenesis to Pancreatic B Cell Acid and Glucose-6-Phosphatase.
Sydney S. Lazarus (introduced by Bruno W. Volk).

93. Effect of Glucagon on Hepatic Blood Flow and Metabolism in Unanesthetized Dog.
W. C. Shoemaker and *T. B. Van Itallia* (introduced by Jay J. Gold).

TO BE READ BY TITLE

See Abstracts 94–170

NOTICE

Dr. Ernest Knobil will serve as Acting Managing Editor of ENDOCRINOLOGY while the Managing Editor is on leave-of-absence during the last six months of 1959. Manuscripts and correspondence should continue to be addressed to ENDOCRINOLOGY, Editorial Office, *25 Shattuck Street, Boston 15, Massachusetts.*